

Molecular genetics of X-linked cone-rod dystrophy and Åland Island eye disease

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To my late father, Pertti

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by their Roman numerals. In addition some unpublished data (U) are presented.

- I Jalkanen R, Demirci FY, Tyynismaa H, Bech-Hansen T, Meindl A, Peippo M, Mäntyjärvi M, Gorin MB, Alitalo T. A new genetic locus for X linked progressive cone-rod dystrophy. *Journal of Medical Genetics* 40:418-423, 2003
- II Jalkanen R, Mäntyjärvi M, Tobias R, Isosomppi J, Sankila E-M, Alitalo T, Bech-Hansen NT. X linked cone-rod dystrophy, CORDX3, is caused by a mutation in the *CACNA1F* gene. *Journal of Medical Genetics* 43:699-704, 2006
- III Jalkanen R, Bech-Hansen NT, Tobias R, Sankila E-M, Mäntyjärvi M, Forsius H, de la Chapelle A, Alitalo T. A novel *CACNA1F* gene mutation causes Åland Island eye disease. *Investigative Ophthalmology & Visual Science* 48:2498-2502, 2007

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ABBREVIATIONS

AIED	Åland Island eye disease	NMD	nonsense-mediated mRNA decay
AMD	age-related macular degeneration	OPL	outer plexiform layer
BHK-21	baby hamster kidney cells	ORF	open reading frame
bp	base pair	PAGE	polyacrylamide gel electrophoresis
CACNA1F	L-type calcium channel α_{1F} -sub-unit gene	PCR	polymerase chain reaction
CD	cone dystrophy	PTC	premature termination codon
cDNA	complementary deoxyribonucleic acid	rAAV2	recombinant adeno-associated virus 2
cGMP	cyclic guanosine monophosphate	RetNet	Retinal Information Network
cM	centiMorgan	RFLP	restriction fragment length polymorphism
CORDX	X-linked cone-rod dystrophy	RNA	ribonucleic acid
COS-1	African green monkey kidney cells	RP	retinitis pigmentosa
CRD	cone-rod dystrophy	RPE	retinal pigment epithelium
CSNB	congenital stationary night blindness	RT-PCR	reverse transcriptase polymerase chain reaction
CSNBX	X-linked congenital stationary night blindness	SNP	single nucleotide polymorphism
DNA	deoxyribonucleic acid	SSCP	single-strand conformation polymorphism (also known as SSCA)
ENCODE	Encyclopedia of DNA elements	STGD	Stargardt disease
ERG	electroretinogram	VDCC	voltage-dependent calcium channel
GFP	green fluorescent protein	wt	wild-type
HEK-293	human embryonic kidney cells		
HGP	Human Genome Project		
HVA	high voltage-activated		
IPL	inner plexiform layer		
kb	kilobase		
LCA	Leber congenital amaurosis		
LOD	logarithm of odds		
LVA	low voltage-activated		
Mb	megabase		
mRNA	messenger ribonucleic acid		
NCBI	National Center for Biotechnology Information		

Standard one-/three-letter codes for amino acids and nucleic acid bases are used.

ABSTRACT

X-linked cone-rod dystrophy is a retinal disease characterized by progressive loss of cone and rod photoreceptor functions. Affected males show reduced visual acuity, photophobia, myopia, color vision defects, central scotomas, and variable fundus changes. The disease is genetically heterogeneous and two disease loci, *CORDX1* and *CORDX2*, were known prior to the present thesis work. *CORDX1*, located on chromosome Xp21.1-11.4, is caused by mutations in the *RPGR* gene, which was originally identified as the causative gene for RP3 type retinitis pigmentosa. *CORDX2* is located on Xq27-28 but the causative gene is still unknown.

Åland Island eye disease (AIED), also known as Forsius-Eriksson syndrome, or Forsius-Eriksson type ocular albinism, is a retinal disease characterized by fundus hypopigmentation, decreased visual acuity, nystagmus, astigmatism, red color vision defect, myopia, and defective night vision. AIED shares similarities with another retinal disease, congenital stationary night blindness (CSNB2). Mutations in the L-type calcium channel α_{1F} -subunit gene, *CACNA1F*, are known to cause CSNB2, as well as AIED-like disease. The disease locus of the original AIED family maps to the same genetic interval as the *CACNA1F* gene, but first sequencing attempts in the patients of the original AIED family have failed to reveal any *CACNA1F* mutations.

The aims of this study were to map the disease gene in a large Finnish family with X-linked cone-rod dystrophy, to identify the disease

genes behind the cone-rod dystrophy and AIED phenotypes, and to determine the functional consequences of the mutations identified. With the help of linkage and haplotype analyses, we could localize the disease gene of the Finnish cone-rod dystrophy family to the Xp11.4-Xq13.1 region, and thus establish a new genetic X-linked cone-rod dystrophy locus, *CORDX3*. Mutation analyses of candidate genes revealed a novel *CACNA1F* gene mutation, IVS28-1 GCGTC>TGG, in *CORDX3* patients. Surprisingly, a 425 bp *CACNA1F* gene deletion, comprising exon 30 and parts of the adjacent intronic regions, was identified in AIED patients. An additional Finnish patient with a CSNB2-like phenotype was later included in the mutation analysis, which led to the identification of a third *CACNA1F* gene mutation, IVS16+2T>C. All three novel mutations altered splice sites of the *CACNA1F* gene, and resulted in defective pre-mRNA splicing. Several aberrant splice variants were observed predicting different sized deletions or premature termination codons (PTCs) to the calcium channel α_{1F} -subunit protein. Altered or absent channel function is thus the probable disease mechanism resulting from these mutations. The analyses of *CACNA1F* mRNA also revealed novel alternative wt splice variants. These variants predicted PTCs or deletions in the channel protein, suggesting that alternative splicing has an important role in enhancing channel diversity and regulating the overall expression level of the channel.

Several researchers have studied the functional consequences of *CACNA1F* mutations by over-

expressing mutant channels in kidney cells or *Xenopus laevis* oocytes. In this study, however, overexpression of even the wt channel in kidney cells resulted in misfolding of the channel protein and consequent formation of protein aggregates. Thus, other approaches are needed to reveal the actual functional consequences of the observed *CACNA1F* mutations.

The phenotypic spectrum associated with *CACNA1F* mutations is wide. Distinct mutations may lead to several different retinal phenotypes, including 1) CSNB2, 2) CSNB2 with atypical retinal atrophy and visual field defects, 3) atrophy of the retina and the optic discs, 4) severe CSNB2-like phenotype with intellectual disability and symptoms in female carriers, and 5) AIED-like phenotype. In this study, mutations were also identified in patients with CORDX3 and AIED. All these eye disorders have a few symptoms in common, suggesting that thorough clinical examination, together with genetic testing, is necessary to reach a correct diagnosis.

REVIEW OF THE LITERATURE

1 Disease gene identification

1.1 The Human Genome Project

Detailed information of the human genome is crucial for our understanding of human biology and the great number of disorders that can affect us. In order to understand the structure and function of the human genome, an international research program, the Human Genome Project (HGP, www.genomics.energy.gov), was launched in 1990 by the National Institutes of Health and the U.S. Department of Energy. The main goals of the project were to determine the complete DNA sequence of the human genome, identify all the genes encoded by human DNA, and store this information in public databases available to researchers around the world.

The estimated size of the human genome is 3.2 gigabases (Gb), of which about 2.95 Gb is euchromatic, *i.e.* gene-rich regions, as opposed to heterochromatic regions (Baltimore 2001). The first steps to reveal the DNA sequence of the euchromatic portion of the genome were taken in the mid 1990's, when comprehensive genetic and physical maps of the human genome were constructed, providing key tools for the identification of disease genes (Dib et al. 1996; Gyapay et al. 1994; Hudson et al. 1995). The first entire human chromosome sequence was published in December 1999 for the smallest chromosome, chromosome 22 (Dunham et al. 1999). In February 2001, the first draft sequence, covering 90% of the euchromatic regions of the human genome, was reported at the same time by the HGP

(Lander et al. 2001) and a private company, Celera Genomics (Venter et al. 2001). In April 2003, special issues of *Nature* and *Science* presented the completion of the HGP, which had accomplished the sequencing of nearly the entire euchromatic portion of the human genome with almost 100% accuracy. The complete sequences for the remaining chromosomes were published subsequently, and finally, in May 2006, the entire euchromatic sequence of the last unsequenced chromosome, chromosome 1, was published (Gregory et al. 2006). Sequencing of the entire genome from a number of individuals revealed 3.7 million single nucleotide polymorphisms (SNPs), which serve as valuable tools in the identification of disease genes.

The most surprising finding of the HGP was that the number of human genes appeared to be significantly lower than previous estimates, which ranged from approximately 35,000 to as many as 140,000 (Aparicio 2000). According to the latest estimates, the human genome contains 20,000-25,000 protein-coding genes, which cover less than 2% of the euchromatic DNA (International Human Genome Sequencing Consortium 2004). At the moment, the Ensemble databank lists 21,541 protein-coding genes and 4421 RNA genes (www.ensembl.org). The rest of the DNA consists to a great extent of various types of repeat sequences, including transposon-derived repeats, pseudogenes, simple sequence repeats, segmental duplications, and blocks of tandemly repeated sequences such as centromeres, telomeres, and the short arms of acrocentric chromosomes, which together account for at least 50% of the human genome (Lander et al. 2001). Other regions include

regulatory regions as well as regions of as yet unknown features (Birney et al. 2001).

1.2 The ENCODE project

The HGP provided accurate DNA sequences for each of our 24 chromosomes. Knowledge about protein-coding regions of the human genome, however, remains incomplete. The understanding of both non-protein-coding transcripts and functional, noncoding genomic elements remains even poorer (Birney et al. 2007). The ENCODE (ENCyclopedia Of DNA Elements, www.genome.gov/ENCODE) project was launched in September 2003 by the National Human Genome Research Institute in order to identify functional elements in the human genome, including protein-coding genes, non-protein-coding genes, transcriptional regulatory elements, sequences that mediate chromosome structure and dynamics, and as yet undefined functional sequences (ENCODE Project Consortium 2004). ENCODE is being conducted in three phases: a pilot phase, a technology development phase, and a production phase. The pilot phase, in which the ENCODE Consortium is evaluating strategies for identifying various types of genomic elements, is focused on a selected 30 megabases (Mb) that encompasses 1% of the human genome. The goal of the pilot phase is to test a set of procedures that can be applied to characterize the remaining 99% of the human genome.

Results from the pilot phase were published in June 2007 (Birney et al. 2007). One of the major findings of this project was that protein-coding genes seem to be transcriptionally much more complex than previously thought.

Instead of the traditional view that many genes have several alternatively spliced transcripts that code for alternative proteins, the ENCODE data suggests that, in addition, genes may produce other coding or regulatory transcripts that include sequences from both strands or even from neighboring genes. These findings prompted researchers to update the definition of the gene from “the DNA segment that contributes to phenotype/function” to “union of genomic sequences encoding a coherent set of potentially overlapping functional products” (Gerstein et al. 2007). Also, large sections of DNA not annotated as known genes seem to be transcribed into RNA (Birney et al. 2007). All these noncoding RNA products do not necessarily have a function themselves, but transcription of a certain region may be important for chromatin accessibility during DNA replication or binding of transcription factors (Gerstein et al. 2007).

1.3 Positional cloning of disease genes

The majority of the disease genes discovered in the early days of molecular genetics, during the 1980's, were identified by the *functional cloning* approach (Collins 1992). In this strategy, knowledge about the chromosomal location of the disease gene is not needed, and a gene is cloned solely based on the exact information about the basic biochemical defect. As the defective protein is known, a cDNA sequence of the corresponding gene can be produced following protein sequencing or by purifying the corresponding mRNA by immunoprecipitation, and then trapping the appropriate clone from a cDNA library. This strategy was successfully used for example in the identification of the disease gene for phenylketonuria (Robson

et al. 1982). Because detailed information of the biochemical defect is rarely available, new strategies were developed for more efficient disease gene discovery.

Positional cloning is a method of gene discovery where a disease gene is isolated based on its chromosomal location without any prior information on the gene function (Collins 1992; Collins 1995). In some cases the presence of chromosomal aberrations may help to locate the disease gene (Ballabio 1993). The mode of inheritance may also define the candidate region to a certain chromosome, as in the case of X-linked diseases. The Duchenne muscular dystrophy (*DMD*) gene was one of the first disease genes isolated by positional cloning method, taking the advantage of both the X-linked inheritance and chromosomal deletions observed in patients (Monaco et al. 1986). In most cases, however, the location of the gene of interest is defined by linkage analysis utilizing families with affected individuals. In traditional positional cloning, the steps following linkage analysis were usually very laborious, including construction of a continuum of overlapping genomic clones for the candidate region, isolation of DNA from the clones, identification of transcripts, and cloning the cDNAs. Nowadays, the availability of DNA sequence and tools for sequence analysis in public databases has revolutionized traditional positional cloning, allowing the use of the *positional candidate gene method*. Once the region of interest has been identified by linkage analysis, all the genes mapping to this region may serve as potential candidate genes (Collins 1995). Information on gene function, expression profiles, or homologous genes in other organisms may point to the most

attractive candidate genes, which then can be screened for disease-associated mutations. Several methods for mutation detection exist, showing variability in both sensitivity and expense. Following rapid development of automated DNA sequencing techniques, direct sequencing is often the most favorable choice for mutation detection, provided that the number of samples is reasonable. The positional candidate gene strategy has been successful in identifying a number of disease genes, including the disease genes identified in this thesis.

1.4 Linkage and linkage disequilibrium analyses

Linkage analysis is a powerful tool in defining the chromosomal locations of disease genes, and it is often the first step in disease gene identification. Linkage analysis is based on the observation that loci that reside physically close to each other on the same chromosome only rarely separate from each other due to crossing over (*i.e.* a recombination event) during meiosis (Lathrop et al. 1984; Pulst 1999). When two loci are located on distinct chromosomes or on the same chromosome but far away from each other, their alleles will be inherited independently, according to Mendel's laws. On the other hand, when two loci are situated close enough on the same chromosome, their alleles will be inherited together from one generation to the next more often than would be expected by chance, and these loci are said to be linked (de la Chapelle et al. 1994).

The frequency of observed recombinations (the recombination fraction, θ) is used to calculate the genetic distance between two loci (Pericak-

Vance 1998). The unit for genetic distance is the centiMorgan (cM), which corresponds to a recombination fraction of 0.01 (1%). When the recombination fraction exceeds 0.05-0.1 (5-10%), it does not correlate directly with cMs, because double crossing-overs are likely to occur. Several map functions are available to calculate genetic distances corresponding to certain recombination fractions (Haldane 1919; Kosambi 1944). If the recombination fraction exceeds 0.5 (50%), two loci are inherited independently, as for those located on distinct chromosomes, and linkage is absent.

The total genetic length of the human genome is ~3700 cM, and, on average, 1 cM corresponds to a physical distance of 1 Mb (Pulst 1999). Because recombinations occur more often during female than male meioses, the genetic map in females is longer than in males (Dib et al. 1996). Recombinations are also most frequent in telomeric and subtelomeric regions. According to recent studies, recombination events occur nonrandomly at specific 1-2 kilobase (kb) recombination “hot spots”, whereas the surrounding regions are “cold” (Kauppi et al. 2004). The nature of recombination hot spots is not fully understood, and hot spots located in different genomic regions share no obvious sequence similarities with each other. Chromatin structure seems, however, to play a partial role in the determination of recombination sites.

The statistical significance of linkage is measured by the logarithm of odds (LOD) score (Morton 1955). The lod score (Z) is a 10th base logarithm of the likelihood that two loci are linked at a certain recombination fraction divided by the likelihood that the two

loci are unlinked ($\theta=0.5$). At the maximum lod score value, the corresponding recombination fraction gives the most likely genetic distance between the loci. A lod score of 3 or higher (odds ratio 1000:1 favoring linkage) is generally accepted as evidence of linkage, whereas at lod scores of -2 or less linkage is excluded.

In genetically homogenous populations, the candidate region determined by linkage analysis (typically several Mbs), is often narrowed down by linkage disequilibrium analysis (Terwilliger 1995; Xiong and Guo 1997). Linkage disequilibrium refers to the situation that specific alleles at two loci occur together more often at the population level than would be expected from the allele frequencies (Pulst 1999). The power of linkage disequilibrium comes from the utilization of historical recombinations from past generations (Jorde 1995). When a mutation is first introduced into a population, it appears in a single chromosome containing a certain series of marker alleles, called a haplotype. Throughout the generations, recombinations occur between the disease gene and the marker loci, and the common haplotype flanking the mutation becomes smaller. The size of the common haplotype associated with the disease mutation depends on two factors: recombination frequency and the number of generations since the mutation was introduced to the population. Linkage disequilibrium can be expected only if the disease mutation in a population is of single-origin (a new mutation or immigration of the mutation carrier), a so called founder mutation (de la Chapelle and Wright 1998). Other important determinants of a population that can be studied by linkage disequilibrium mapping include a relatively small

number of founder individuals, the expansion of the population by growth rather than by immigration, and large enough population size to provide affected individuals to study. The Finnish population is a well-known example of a population which fulfills these criteria, and linkage disequilibrium has been successfully used in identifying the genetic background of many diseases belonging to the Finnish disease heritage, such as diastrophic dysplasia (Hästbacka et al. 1994), infantile neuronal ceroid lipofuscinosis (INCL) (Järvelä 1991; Vesa et al. 1995), and progressive myoclonus epilepsy (EPM1) (Pennacchio et al. 1996; Virtaneva et al. 1996).

1.5 Genetic markers

Linkage analysis is based on the segregation of alleles at polymorphic loci (genetic markers) in families with affected individuals. Polymorphisms are defined as DNA sequence differences that are present in over 1% of a given population. Polymorphisms occur most frequently in noncoding regions, but when present in a coding region they do not usually change the amino acid, or alternatively, the amino acid change is silent and the protein function is unaffected (Jalanko et al. 1996). The most useful polymorphisms in linkage studies are abundant, easy to genotype, and have high degree of heterozygosity.

The first polymorphic markers utilized in linkage analyses were restriction fragment length polymorphisms (RFLPs). RFLPs are caused by a single nucleotide change in the specific recognition site of restriction endonucleases, or by deletions and insertions in the restriction fragments (Botstein et al. 1980). The use-

fulness of RFLPs is limited because of the laborious methodology, and relatively low information content. With the advent of the polymerase chain reaction (PCR) (Mullis et al. 1986), RFLPs were soon replaced by more informative minisatellite (Jeffreys et al. 1985) and microsatellite markers (Weber and May 1989). The disadvantage of minisatellites is that they are nonrandomly dispersed in the human genome, showing preferential localization to the ends of chromosomes (Royle et al. 1988). Microsatellites (di-, tri-, and tetranucleotide repeats), on the other hand, are dispersed relatively equally throughout the genome (Li et al. 2002). Furthermore, they have high allelic diversity and therefore high heterozygosity. Recent interest has, however, turned back to single nucleotide polymorphisms (SNPs). Although SNPs are less informative than microsatellite markers, their abundance and the recent advances in automation of genotyping by novel "chip"- or microarray-based methods (such as those developed by Affymetrix, Illumina, and Sequenom) have made them a powerful tool for gene mapping studies. Other benefits of SNPs include lower *de novo* mutation rates than those of microsatellites, and occurrence in both noncoding and coding regions (Gray et al. 2000).

2 Physiology of vision

Vision has an important role in the understanding of the world around us. Visual perception begins in the back of the eye, where a thin layer of neural cells, called the retina, gathers and processes information about light, colors, shapes, and movements of the outside world, to be transferred to and interpreted by the brain.

2.1 Retinal development

The vertebrate eye is a complicated structure, comprising a number of different tissue types. During vertebrate embryogenesis the eye develops as a consequence of interactions between the surface ectoderm and embryonic forebrain (Wong 2006). The retina forms as a forebrain evagination, the optic vesicle, folds inward to form an optic cup. The outer wall of the optic cup becomes the retinal pigment epithelium (RPE), whereas cells in the inner wall differentiate into the neural retina. The optic stalk, connecting the optic vesicle to the developing brain, eventually becomes the optic nerve.

In humans, eyes start to develop three weeks after fertilization (Mann 1964). By embryonal week 30, cell proliferation is complete throughout the retina (Provis et al. 1985a). Cell differentiation occurs in two phases: ganglion cells, horizontal cells, and cone photoreceptors are generated in the early phase, whereas amacrine cells, bipolar cells, rod photoreceptors, and Müller glia cells differentiate in the later phase (Rapaport 2006). Retinal neurogenesis occurs in concert with programmed cell death: approximately 70% of the ganglion cells generated during development are lost in fetal life (Provis et al. 1985b). The rate of developmental cell death among other retinal cells is less clear. Retinal differentiation is not fully completed during fetal life, and maturation continues even several months after birth (Yuodelis and Hendrickson 1986).

2.2 Retinal structure

The neural retina is arranged in a typical layered structure found in all vertebrates (Figure 1) (Dowling 1987). Photoreceptors constitute the outermost layer, hence, light must travel through all the other retinal layers to reach the light-sensitive cells. The two major types of photoreceptors are rods and cones. Rod photoreceptors are extremely sensitive to light, and thereby mediate vision in dim light, whereas cones are specialized to operate in bright light, and are responsible for high resolution color vision. The proportions of photoreceptors vary widely between different retinal regions, and also between species. The Chicken (*Gallus domesticus*) retina, for example, is cone-dominated (Trevino et al. 2005), whereas the frog (*Xenopus laevis*) has roughly equal numbers of rods and cones (Chang and Harris 1998). In human retina, rods are the predominant receptor type (Curcio et al. 1990). Cone density is highest in the foveal region, located in the center of the macula lutea, and falls steeply outside the fovea. The foveola in the center of the fovea, responsible for the highest visual acuity, is a rod-free zone containing only cones. The rod cells are the predominant receptor type outside the fovea with highest density in the ring-like area at a distance of about 3-5 mm from the foveola (Jonas et al. 1992). The optic disc, where ganglion cell axons exit the eye to form the optic nerve, does not contain any photoreceptors, and is therefore also referred to as “the blind spot” (Awater et al. 2005).

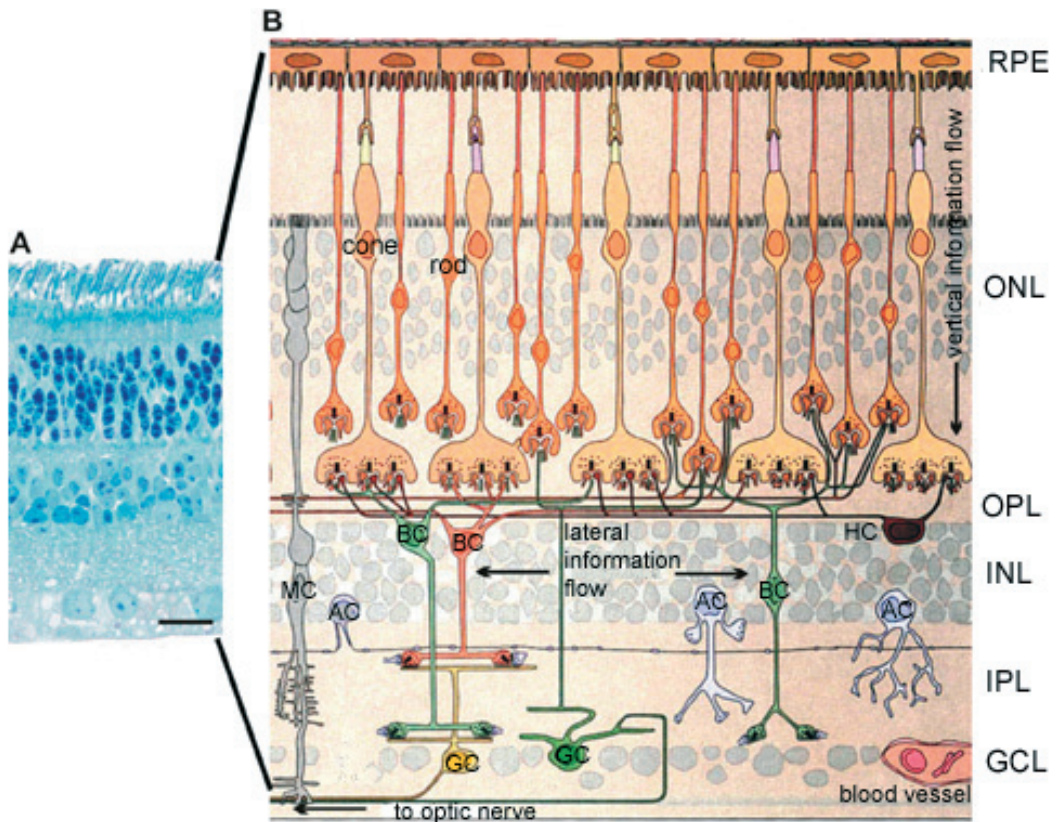


Figure 1. Retinal anatomy and the main cell types. All vertebrate retinas are composed of three layers of nerve cell bodies (ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer), and two layers of synapses (OPL, outer plexiform layer; IPL, inner plexiform layer). The photoreceptors, rods and cones, convert the light stimulus into electrical and chemical signals, and transfer the information to bipolar cells (BC). Bipolar cells are in contact with ganglion cells (GC) whose axons form the optic nerve connecting the retina to the brain. Lateral information flow is mediated by horizontal cells (HC), which operate at the OPL, and amacrine cells (AC), which function at the IPL. Müller cells (MC) extend through the entire retina providing functional and structural support. In reality, the retina is a much more complex structure, and many of the main cell types contain several subtypes each with specific functions (Masland 2001). The retinal pigment epithelium (RPE) just behind the neural retina provides metabolic support to the photoreceptors. Image adapted from University of Melbourne, Department of Anatomy and Cell biology web pages (www.anatomy.unimelb.edu.au/researchlabs/rees/images/retina.jpg, Copyright © The University of Melbourne 1994-2007).

2.3 Rod and cone photoreceptors and phototransduction

Rods and cones have the same basic structure: an outer segment and an inner segment connected by a narrow connecting cilium (Fig 2A). The outer segments face the RPE and contain stacks of membrane disks filled with visual pigments. The visual pigments consist of a protein part, opsin, and a vitamin A derived chromophore, retinal. The visual pigment in rod photoreceptors is rhodopsin (Hargrave et al. 1983). Cones have different visual pigments called cone opsins. Humans have three different

cone opsins sensitive to different wavelengths of light (Nathans et al. 1986). L (or red) cones are maximally sensitive to long wavelengths of light, M (or green) cones to medium wavelengths, and S (or blue) cones to short wavelengths of light. The absorption of light photons by visual pigment molecules leads to a series of events, called phototransduction (Figure 2B), which eventually leads to closure of cation-specific channels and subsequent hyperpolarization of the plasma membrane and the signaling of

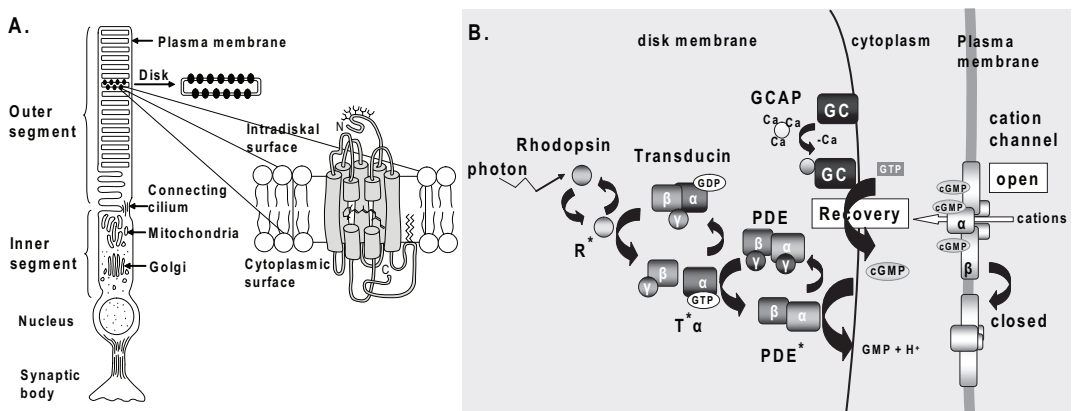


Figure 2A. Rod cell and rhodopsin molecule structure. The inner segment contains the metabolic machinery of a rod cell, whereas the outer segment is filled with rhodopsin-containing membrane disks. Rhodopsin is a member of G-protein coupled receptor family. It contains a protein part with seven transmembrane helices, and a chromophore, 11-cis-retinal. Image adapted from Hargrave and coworkers (1993). **B.** Details of phototransduction. The absorption of a light photon by rhodopsin leads to isomerization of the chromophore 11-cis-retinal to the all-trans form, and subsequent activation of rhodopsin. The activated rhodopsin (R^*) stimulates G-protein, transducin, and causes the replacement of bound guanosine diphosphate (GDP) with guanosine triphosphate (GTP) in the transducin α -subunit. The α -subunit-GTP complex ($T^*\alpha$) then activates phosphodiesterase (PDE) by removing the inhibitory γ -subunits. Activated PDE (PDE^*) catalyzes the hydrolysis of cyclic guanosine monophosphate (cGMP), which leads to closure of cGMP-activated cation channels and hyperpolarization of the cell membrane. After a photoresponse, the decreased intracellular calcium levels allow Ca^{2+} ions to dissociate from guanylate cyclase-activating protein (GCAP). Ca^{2+} -free GCAP then activates guanylate cyclase (GC), which synthesizes cGMP for reopening the cation channels. A number of other proteins are required to deactivate other steps of the phototransduction cascade, and return rhodopsin to its inactive state, ready for absorption of another photon. Image adapted from Polans and coworkers (1996).

second-order neurons by neurotransmitters (Pepe 2001).

The interaction of photoreceptors with the RPE is essential for visual function (Strauss 2005). The RPE is composed of a single layer of cells that are densely packed with melanin pigment granules. It delivers nutrients to the photoreceptors, and transports ions, water and metabolic end products to the blood. To maintain photoreceptor excitability, RPE re-isomerizes all-*trans*-retinal back into 11-*cis*-retinal, stabilizes the ion composition of the subretinal space, and phagocytizes the tips of continuously renewing photoreceptor outer segments. The RPE also secretes growth factors that help to maintain the structural integrity of photoreceptors. Furthermore, it protects the retina against light-generated oxygen reactive species.

2.4 Evaluating retinal function

A recording of electrical activity of retinal cells is called an electroretinogram (ERG). In ERG measurements, a low-resistance electrode is placed on the corneal surface and a reference electrode elsewhere on the head. The eye is then stimulated with a flash of light and the resulting voltage-changes are recorded with an oscilloscope. ERG can be used for evaluating retinal and visual function, and it is a very useful tool in clinical diagnosis. ERG is composed of three major components (Dowling 1987). The *a-wave*, which has negative polarity, originates from rod and cone photoreceptors. The following positive *b-wave* reflects the activity of the inner retinal neurons, mainly depolarizing bipolar cells. With repetitive bright flashes oscillatory potentials, which originate from the

amacrine cells, appear in the *b-wave*. A second, much slower positive component, the *c-wave*, is generated by the RPE. ERG can be measured in different conditions to separate rod- and cone-derived responses: under scotopic (*i.e.* dark-adapted) conditions ERG reflects the function of rod photoreceptors whereas under photopic (*i.e.* light-adapted) conditions rods are bleached and only the function of cones is retained.

3 Inherited retinal diseases

Taking into account the highly complex and sophisticated structure of the eye, it is not surprising that the eye is one of the most common sites of genetic diseases (Gregory-Evans and Bhattacharya 1998). Diseases of the retina, such as age-related macular degeneration (AMD) and diabetic retinopathy, are especially important, being the leading causes of blindness in the Western world. Inherited retinal diseases, on the other hand, are the most common cause of vision loss among the working population in Western countries. It is estimated that ~1‰ of the people worldwide suffer from vision loss due to inherited retinal diseases, also known as retinal degenerations or retinal dystrophies. In Finland this adds up to over 5000 affected individuals (www.retina.fi). The severity of these diseases varies from partial vision loss to total blindness. A growing body of data suggests that genetic factors also play a role in the development of the most common retinal disease, AMD (Patel et al. 2007).

3.1 Inheritance

To date, nearly 200 mapped loci, including 138 cloned genes for inherited retinal diseases, have been identified (Retinal Information Network, RetNet, www.sph.uth.tmc.edu/Retnet/home.htm). The mode of inheritance can be autosomal dominant or recessive, X-linked dominant or recessive, digenic, mitochondrial, or complex. The genes associated with inherited retinal diseases are involved in many different cellular processes and functions, including visual transduction, retinoid cycle, regulation of gene expression, splicing, cellular trafficking, and metabolic and structural functions (Gregory-Evans and Bhattacharya 1998; Maubaret and Hamel 2005; Travis et al. 2007). The majority of the genes are retina-specific or retina-enriched, but ubiquitously expressed genes may also cause “pure” retinal phenotypes (Blackshaw et al. 2001). In some cases, even genes that are not expressed in the retina may cause a retinal phenotype, as in the case of retinol binding protein, RBP4, which delivers retinol from the liver stores to the peripheral tissues (Seeliger et al. 1999).

3.2 Exceptional heterogeneity

A striking feature in retinal diseases is the involvement of exceptional genetic, allelic, and clinical heterogeneity. Mutations in many different genes may cause the same disease, while different mutations in the same gene may cause different diseases. Furthermore, clinical features may be variable even among family members who carry the same mutation (Sullivan and Daiger 1996). A good example of genetic heterogeneity is retinitis pigmentosa (RP), with at least 47 mapped loci or identified

genes responsible for the phenotype (RetNet web page). Many of the disease genes also show vast allelic heterogeneity; for example mutations in *ABCA4* lead to Stargardt disease (STGD) (Allikmets et al. 1997b) but may in addition cause RP (Cremers et al. 1998; Martinez-Mir et al. 1998), fundus flavimaculatus (FFM, a late-onset subtype of STGD) (Rozet et al. 1998), AMD (Allikmets et al. 1997a), and cone-rod dystrophy (CRD) (Cremers et al. 1998).

3.3 Development of clinical therapies

No effective treatments for inherited retinal diseases exist at the moment. The expanding knowledge about the molecular and genetic basis of these diseases is, however, continually improving the prospects for rational treatments. Active research is ongoing around a variety of intriguing treatment methods, including retinal cell transplantation and stem cells, artificial retinal implants, as well as growth factors and pharmaceutical therapies (www.blindness.org). The most promising advance in the past few years has, however, been made in the development of gene therapy approaches. The eye can be considered as a good target for gene therapy, because it is accessible, easily examined by ophthalmoscopy, and the blood-retinal and blood-aqueous barriers help to target the vectors to the desired area without spreading out of the eye (Ali et al. 1997). The most efficient gene delivery vehicles for treatment of retinal diseases are recombinant adeno-associated virus 2 (rAAV2) vectors, which achieve efficient and stable gene transfer to RPE, photoreceptors, and ganglion cells (Auricchio and Rolling 2005). Convincing results were obtained recently when RPE65-/-

biard dogs, with a retinal phenotype resembling that of Leber congenital amaurosis (LCA) in humans, were treated with rAAV2/4 vector containing a human *RPE65* promoter and cDNA. The targeted gene transfer in these dogs led to the stable restoration of normal retinal function and vision-dependent behavior (Le Meur et al. 2007). Clinical trials in humans have just begun, and may soon provide aid for children who have been born blind due to LCA, caused by *RPE65* mutations (www.clinicaltrials.gov).

3.4 Disease types

The genetic defect in inherited retinal diseases usually results in loss of photoreceptors (Travis 1998). According to photoreceptor loss, these diseases can be broadly classified into two types: those that initially affect rods in the peripheral retina, and those that initially affect cones in the central retina (Gregory-Evans and Bhattacharya 1998). In many disorders, however, both rod and cone systems are involved already in the early stages of the disease (Bird 1995). Diseases that start with peripheral vision loss are called rod-cone dystrophies. The most common disease type is RP, which is characterized by primary degeneration of rod photoreceptors, with secondary degeneration of cones (Hamel 2006). The earliest symptom is night blindness resulting from predominant rod involvement. As the disease progresses, initial peripheral vision loss evolves to ring shaped scotoma and eventually to tunnel vision. In later stages color vision may also be affected, and visual acuity decreases due to cone involvement. In contrast to rod-cone dystrophies, central retinal dystrophies that include maculopathies as well as cone

dystrophies (CDs) and cone-rod dystrophies reflect the predominant involvement of cones, which leads to decreased visual acuity and loss of the central visual field, while peripheral vision is retained for a longer time (Gregory-Evans and Bhattacharya 1998). Most frequently, retinal dystrophies are nonsyndromic, but there are some syndromes in which retinal degeneration is a characteristic feature. For example, in Usher syndromes sensorineural deafness is associated with typical RP (Boughman et al. 1983). Another example is Bardet-Biedl syndrome (BBS), which is characterized by retinal dystrophy, postaxial polydactyly, central obesity, mental retardation, hypogonadism, and renal dysfunction (Beales et al. 1999).

3.5 Cone- and cone-rod dystrophies

Disorders with cone cell dysfunction can be classified into two main categories: stationary and progressive disorders. Stationary cone dystrophies, better described as stationary cone dysfunction syndromes, are usually present already shortly after birth, and display normal rod function (Michaelides et al. 2004). Progressive cone dystrophies, on the other hand, appear in childhood or early adulthood, and may show variable extents of rod involvement (Michaelides et al. 2006). Thereby, progressive cone dystrophies show marked overlap with cone-rod dystrophies.

Cone dystrophies and cone-rod dystrophies (prevalence 1:40,000) are a subgroup of inherited retinal degenerations characterized by initial loss of the cone system followed by variable degrees of rod degeneration (Hamel 2007; Michaelides et al. 2006). These diseases are usually discovered at school

age due to decreased visual acuity that does not significantly improve with spectacles. Photophobia and color vision disturbances also appear at early stages resulting from primary cone involvement. In visual field tests patients show central scotomas whereas the periphery is spared. Fundus examination usually reveals pigment deposits, macular atrophy, and pale optic discs. ERG shows affected cone or cone-rod responses. Due to initial loss of central vision patients try to project images onto the less damaged parafoveal region, resulting in a characteristic deviated gaze. As the disease progresses, visual acuity continues to decrease along with the loss of peripheral vision. Additionally, nystagmus and night blindness become more apparent. These diseases are clinically heterogeneous, and even family members with same mutation may show marked variation in disease severity (Downes et al. 2001; Michaelides et al. 2005). Histopathologic findings indicate regional loss or reduced numbers of photoreceptors with shortened outer segments (Demirci et al. 2005; Rabb et al. 1986). RPE cells show accumulation of lipofuscin granules, and are atrophic or absent in the macular area.

Like other inherited retinal degenerations, CDs and CRDs also show a vast genetic and allelic heterogeneity. The disease can be inherited in autosomal dominant, autosomal recessive, or X-chromosomal recessive manners. At least 22 genes are responsible for CD or CRD phenotypes, 20 on autosomes and 2 on the X-chromosome (Table 1). Mutations in most of these genes may also lead to other inherited retinal degenerations.

3.6 X-linked cone- and cone-rod dystrophies

3.6.1 *Clinical findings*

Patients with X-linked recessive cone- or cone-rod dystrophy (CORDX, formerly COD) show typical features of cone- and cone-rod dystrophies, including reduced visual acuity, photophobia, color vision defects, central scotomas, and affected cone or cone-rod responses in ERG (Brown et al. 2000; Hong et al. 1994; Jacobson et al. 1989; Meire et al. 1994; Mäntyjärvi et al. 2001; Pinckers and Timmerman 1981). Fundus findings are nonspecific ranging from normal or subtle granularity of the macula, to bull's eye lesions and central geographic atrophy of retinal pigment epithelium. In some patients a tapetal-like sheen of the retina is also present (Brown et al. 2000; Heckenlively and Weleber 1986; Jacobson et al. 1989). In addition to the general features of cone and cone-rod dystrophies mentioned above, patients with X-linked forms of the disease often show moderate or high myopia. Nystagmus is usually absent. The disease begins in most cases within the first two decades of life and progresses gradually, however, the phenotypic expression of the disease varies with respect to age of onset and severity of symptoms (Brown et al. 2000; Hong et al. 1994; Jacobson et al. 1989; Mäntyjärvi et al. 2001). Additionally, female carriers show variable expression of the disease, ranging from clinically asymptomatic to mild impairment of visual acuity, light sensitivity, and slight abnormalities in color vision, ophthalmoscopy, and ERG (Brown et al. 2000; Hong et al. 1994; Jacobson et al. 1989; Meire et al. 1994; Mäntyjärvi et al. 2001).

Table 1. Identified genes and mapped loci responsible for cone- and cone-rod dystrophies

Gene symbol	Protein product	Locus	Protein function	Associated phenotypes	References
<i>ABCA4</i>	ATP-binding cassette, subfamily A, member 4	<i>CORD3</i> 1p22.1-p21	transporter, retinoid metabolism	ar CRD, STGD, FFM, AMD, RP	1-6
-	-	<i>CORD8</i> 1q23-q24	-	ar CRD	7-8
<i>SEMA4A</i>	Semaphorin 4A	<i>CORD10</i> 1q22	photoreceptor development, T-cell activation	ar CRD, RP	9-11
<i>GUCA1A</i>	Guanylate cyclase activator 1A	<i>COD3</i> 6p21.1	phototransduction	ad CRD, CD	12-14
<i>peripherin/RDS</i>	Retinal degeneration slow protein	6p21.1	adhesion molecule, disk morphogenesis	ad CRD, RP, FFM, MD, fundus albipunctatus, digenic RP	15-23
<i>RIMS1</i>	Rab3-interacting molecule 1	<i>CORD7</i> 6q14	scaffold protein, exocytosis of secretory vesicles	ad CRD	24-25
-	-	<i>CORD9</i> 8p11	-	ar CRD	26
<i>CNGB3</i>	cyclic nucleotide-gated cation channel β subunit	8q21-q22	signal transmission in cones	ar CD, ACHM, MD	27-30
<i>KCNV2</i>	voltage-gated potassium channel subunit	9p24	photoreceptor resting potential	ar CD with supernormal rod ERG	31-32
<i>CACNA2D4</i>	voltage-gated calcium channel $\alpha_2\delta$ subunit,	12p13.33	retinal signal transmission	ar CD	33-34
<i>RDH5</i>	11- <i>cis</i> retinol dehydrogenase 5	12q13.2	retinoid metabolism	ar fundus albipunctatus with or without CD or MD, familial fleck retina with night blindness	35-39
<i>RPGRIP1</i>	RPGR-interacting protein 1	14q11	structural component of the ciliary axoneme	ar CRD, LCA, RP	40-43
<i>GUCY2D</i>	Guanylate cyclase 2D	<i>CORD6</i> 17p13.1	phototransduction	ad CRD, ar LCA, RP	43-46
<i>PITPNM3</i>	Membrane-associated phosphatidylinositol transfer protein 3	<i>CORD5</i> 17p13.1	phototransduction?	ad CD	47-48
<i>AIPL1</i>	Aryl hydrocarbon receptor-interacting protein-like 1	17p13.1	chaperone, retinal protein folding and/or trafficking	ad CRD, ar LCA	49-52
<i>HRG4/UNC119</i>	Human retinal protein 4, unc119 homolog (<i>C. elegans</i>)	17q11.2	retinal signal transmission? T-cell activation	ad CRD	53-54
-	-	<i>CORD4</i> 17q	-	ad CRD with neurofibromatosis 1	55
-	-	<i>CORD1</i> 18q21.1-q21.3	-	ad CRD	56
<i>RAXL1/QRX</i>	Retina and anterior neural fold homeobox-like protein 1	<i>CORD11</i> 19p13.3	photoreceptor gene expression	ad CRD, ARMED	57
<i>CRX</i>	Cone-rod homeobox protein	<i>CORD2</i> 19q13.3	transcription factor	ad CRD, RP, LCA	58-61
<i>RPGR</i>	Retinitis pigmentosa GTPase regulator	<i>CORDX1</i> Xp21.1	Regulation of transport between inner and outer segments?	xl CRD, CD, MD, RP, RP with hearing loss and chronic infections	62-68
-	-	<i>CORDX2</i> Xq27-28	-	xl CD	69

ad, autosomal dominant; ar, autosomal recessive; xl, X-linked; ACHM, achromatopsia; AMD, age-related macular degeneration; CD, cone dystrophy; CRD, cone-rod dystrophy; FFM, fundus flavimaculatus; LCA, Leber congenital amaurosis; MD, macular dystrophy; RP, retinitis

pigmentosa; STGD, Stargardt disease; ERG, electroretinogram; 1, (Allikmets et al. 1997a); 2, (Allikmets et al. 1997b); 3, (Cremers et al. 1998); 4, (Martinez-Mir et al. 1998); 5, (Rozet et al. 1998); 6, (Beharry et al. 2004); 7, (Khaliq et al. 2000); 8, (Ismail et al. 2006); 9, (Kumanogoh et al. 2002); 10, (Rice et al. 2004); 11, (Abid et al. 2006); 12, (Subbaraya et al. 1994); 13, (Payne et al. 1998); 14, (Downes et al. 2001); 15, (Farrar et al. 1991); 16, (Kajiwara et al. 1991); 17, (Travis et al. 1991); 18, (Kajiwara et al. 1993); 19, (Nichols et al. 1993); 20, (Kajiwara et al. 1994); 21, (Apfelstedt-Sylla et al. 1995); 22, (Nakazawa et al. 1996); 23, (Felbor et al. 1997); 24, (Coppola et al. 2001); 25, (Johnson et al. 2003); 26, (Danciger et al. 2001); 27, (Kohl et al. 2000); 28, (Sundin et al. 2000); 29, (Michaelides et al. 2004); 30, (Nishiguchi et al. 2005); 31, (Ottschytsch et al. 2002); 32, (Wu et al. 2006); 33, (Qin et al. 2002); 34, (Wycisk et al. 2006); 35, (Simon et al. 1996); 36, (Yamamoto et al. 1999); 37, (Nakamura et al. 2000); 38, (Nakamura et al. 2003); 39, (Hayashi et al. 2006); 40, (Dryja et al. 2001); 41, (Hong et al. 2001); 42, (Hameed et al. 2003); 43, (Booi et al. 2005); 44, (Shyjan et al. 1992); 45, (Perrault et al. 1996); 46, (Kelsell et al. 1998); 47, (Ocada et al. 2005); 48, (Köhn et al. 2007); 49, (Sohocki et al. 2000a); 50, (Sohocki et al. 2000b); 51, (Liu et al. 2004); 52, (van der Spuy and Cheetham 2004); 53, (Kobayashi et al. 2000); 54, (Gorska et al. 2004); 55, (Kylstra and Aylsworth 1993); 56, (Warburg et al. 1991); 57, (Wang et al. 2004); 58, (Freund et al. 1997); 59, (Swain et al. 1997); 60, (Freund et al. 1998); 61, (Sohocki et al. 1998); 62, (Meindl et al. 1996); 63, (Ayyagari et al. 2002); 64, (Demirci et al. 2002); 65, (Yang et al. 2002); 66, (Zito et al. 2003); 67, (Khanna et al. 2005); 68, (Shu et al. 2005); 69, (Bergen and Pinckers 1997).

3.6.2 Molecular genetics

CORDX is a genetically heterogeneous disorder, with two loci, *CORDX1* (formerly *COD1*, MIM 304020) and *CORDX2* (formerly *COD2*, MIM 300085), localized by the time this thesis work started. *CORDX1*, located on Xp21.1-p11.4 (Bergen et al. 1993; Hong et al. 1994; Seymour et al. 1998), is now known to be caused by mutations in an alternative 3' exon of the *RPGR* gene (Xp21.1, MIM 312610) (Demirci et al. 2002; Yang et al. 2002). *RPGR* was first identified as a causative gene for RP (Meindl et al. 1996; Roepman et al. 1996). The discovery of the new alternative 3' *RPGR* exon, called open reading frame (ORF) 15, revealed a mutational hotspot (Vervoort et al. 2000).

A total of 55% of the *RPGR* mutations identified to date are located in exon ORF15, most likely due to insertions and deletions resulting from the highly repetitive nature of this region (Shu et al. 2007). In addition to RP and CORDX, *RPGR* mutations may also cause atrophic macular dystrophy (Ayyagari et al. 2002), and RP with hearing loss and chronic infections (Zito et al. 2003). The other CORDX locus, *CORDX2*, maps between markers DXS292 and DXS1113 on Xq27-q28, based on linkage analysis in a family with X-linked progressive cone dystrophy (Bergen and Pinckers 1997), but the disease-causing gene remains to be identified.

3.7 Åland Island eye disease (AIED)

3.7.1 *Clinical findings*

Åland Island eye disease (AIED), also referred to as Forsius-Eriksson syndrome, or Forsius-Eriksson type (type II) ocular albinism (MIM 300600), was first described in 1964 in a farmer's family on the Åland Islands in southwestern Finland (Forsius and Eriksson 1964). In addition to the original AIED family, a few families with a similar phenotype, referred to as AIED-like disease, have been described (Carlson et al. 1991; Glass et al. 1993; Hawksworth et al. 1995; Rosenberg et al. 1990). AIED is characterized by fundus hypopigmentation, decreased visual acuity due to foveal hypoplasia, latent nystagmus, astigmatism, red color vision defect, progressive axial myopia, and defective dark adaptation (Carlson et al. 1991; Forsius and Eriksson 1964; Waardenburg 1970). In ERG, both scotopic and photopic functions are affected (Carlson et al. 1991). Except for progression of myopia, and secondary color vision disturbances in older individuals, the disease is considered to be stationary (van Dorp et al. 1985). Female carriers of the disease are clinically normal, except for slight latent nystagmus in some cases (van Vliet et al. 1973).

On the basis of fundus hypopigmentation, AIED was originally regarded as a variant of ocular albinism. The absence of macromelanosomes in skin melanocytes (O'Donnell et al. 1980), and latent nystagmus of extraocular origin (van Vliet et al. 1973) indicated, however, that AIED is distinct from Nettleship-Falls type ocular albinism (MIM 300500). Misrouting of the optic nerve fibers, present in persons

with albinism, was also absent in a patient with AIED (van Dorp et al. 1985). The fundus hypopigmentation in AIED patients presumably results from stretching and thinning of RPE due to the progression of axial myopia (van Dorp et al. 1985).

3.7.2 *Similarity of AIED to congenital stationary night blindness (CSNB2)*

AIED shows notable clinical similarities to another inherited retinal disease, congenital stationary night blindness (CSNB). X-linked CSNB (CSNBX) is a nonprogressive retinal disease characterized by impaired night vision, myopia, nystagmus, strabismus, and reduced visual acuity (Pearce et al. 1990). The expression of the disease is variable, and at least one of the main symptoms is often absent (Boycott et al. 2000; Pearce et al. 1990). ERG in CSNBX patients is of Scubert-Bornschein type (or "negative"), which means that the amplitude of the rod-derived a-wave is larger than the postsynaptic b-wave under scotopic testing conditions (Schubert and Bornschein 1952). Two forms of CSNBX are distinguished based on the ERG findings. The complete type of CSNBX (type 1, CSNB1) is characterized by a lack of detectable scotopic b-waves, whereas in the incomplete type (type 2, CSNB2) the scotopic b-wave is present but diminished (Miyake et al. 1986; Tremblay et al. 1995). The photopic cone responses are also affected, and often more impaired in the incomplete type. Evidence consistent with the clinical subdivision came as the genetic background of CSNBX was resolved. Mutations in the NYX gene (Xp11.4, MIM 300278) are responsible for CSNB1 (MIM 310500) (Bech-Hansen et al. 2000; Pusch et al. 2000), whereas CSNB2

(MIM 300071) results from mutations in the L-type calcium channel α_1 -subunit gene, *CACNA1F* (Xp11.23; MIM 300110) (Bech-Hansen et al. 1998; Strom et al. 1998).

3.7.3 AIED mapping studies

Based on the clinical similarities between AIED and CSNBX, researchers have long discussed whether these two diseases might be allelic or even form a single entity (Krill 1977; Weleber et al. 1989). Linkage studies of the original AIED family supported this idea by localizing the AIED gene locus between markers *MAOA* and DXS559 in the Xp11.3-q13.1 region (Alitalo et al. 1991; Alitalo et al. unpublished linkage data), which overlapped with the *CSNB2* genetic interval. After the genetic background of CSNBX was disclosed, mutation analyses of the *CACNA1F* gene, responsible for CSNB2, were carried out also in AIED families. Sequencing experiments revealed disease-causing mutations in patients with an AIED-like phenotype, however, efforts to reveal *CACNA1F* mutations in patients of the original AIED family were unsuccessful (Wutz et al. 2002).

4 Voltage-dependent calcium channels

Intracellular calcium levels are tightly regulated in eukaryotic cells during normal cell functions. In resting cells, the cytoplasmic free calcium concentration is much lower than in the extracellular environment, mainly due to the Ca^{2+} -impermeable cell membranes, and active transport of Ca^{2+} into intracellular organelles (especially the endoplasmic reticulum) as well as across the plasma membrane (Yamakage

and Namiki 2002). A transient increase in calcium permeability results in Ca^{2+} influx into the cell down its electrochemical gradient. Calcium entry into the cell occurs through ion channels. Ion channels are transmembrane proteins that form selective pores across the lipid bilayer allowing inorganic ions of appropriate size and charge to cross the membrane (Alberts et al. 2002). Channels are not continuously open, instead, they are gated. Based on the stimulus that may open the “gate”, ion channels are subdivided into three main types: *mechanically gated channels*, opened by mechanical stress (Orr et al. 2006), *ligand-gated channels*, opened by the binding of a ligand (Connolly and Wafford 2004), and *voltage-gated channels*, opened by a change in membrane potential (Bezaniila 2005).

The voltage-dependent calcium channels (VDCCs) are a group of voltage-gated channels found in many nonexcitable cells and all excitable cell types (such as neurons and muscle cells), where they serve as transducers of electrical signals into changes in intracellular Ca^{2+} levels (Felix 2005). In the cell, Ca^{2+} acts as a second messenger, activating many crucial intracellular events including muscle contraction, hormone secretion, synaptic transmission, or gene expression, depending on the cell type (Catterall 2000).

4.1 Functional diversity

According to the physiological and pharmacological properties of Ca^{2+} currents, VDCCs are subdivided into multiple classes (Table 2) (Catterall 2000; Catterall et al. 2005). *L-type* channels mediate long-lasting currents, which require strong depolarization for

Table 2. Characteristics of voltage-dependent calcium channel subtypes (modified from Catterall et al. 2005)

Channel	Current	Voltage dependence	α_1 -subunit	Gene*	Specific blocker	Primary tissues	Functions
Ca _v 1.1	L	HVA	α_{1S}	CACNA1S	DHPs	Skeletal muscle	Excitation-contraction coupling
Ca _v 1.2	L	HVA	α_{1C}	CACNA1C	DHPs	Heart, smooth muscle, endocrine cells, neurons	Excitation-contraction coupling, hormone release, gene regulation, synaptic integration
Ca _v 1.3	L	HVA	α_{1D}	CACNA1D	DHPs	Endocrine cells, neurons, heart, cochlea	Hormone release, gene regulation, synaptic regulation, cardiac pacemaking, neurotransmitter release from sensory cells
Ca _v 1.4	L	HVA	α_{1F}	CACNA1F	DHPs	Retina	Neurotransmitter release from photoreceptors
Ca _v 2.1	P/Q	HVA	α_{1A}	CACNA1A	ω -Agatoxin IVA	Neurons, neuroendocrine cells	Neurotransmitter release, dendritic Ca ²⁺ transients, hormone release
Ca _v 2.2	N	HVA	α_{1B}	CACNA1B	ω -Conotoxin GVIA	Neurons, neuroendocrine cells	Neurotransmitter release, dendritic Ca ²⁺ transients, hormone release
Ca _v 2.3	R	HVA/IVA	α_{1E}	CACNA1E	SNX-482	Neurons	Repetitive firing, dendritic Ca ²⁺ transients
Ca _v 3.1	T	LVA	α_{1G}	CACNA1G	none	Neurons, heart, smooth muscle	Pacemaking, repetitive firing
Ca _v 3.2	T	LVA	α_{1H}	CACNA1H	none	Neurons, heart, smooth muscle	Pacemaking, repetitive firing
Ca _v 3.3	T	LVA	α_{1I}	CACNA1I	none	Neurons	Pacemaking, repetitive firing

HVA, high voltage-activated; IVA, intermediate voltage-activated; LVA, low voltage-activated; DHPs, dihydropyridines. *Gene coding for the α_1 -subunit, which determines the channel subtype.

activation and show large single-channel conductance, slow voltage-dependent inactivation, regulation by cAMP-dependent phosphorylation, and sensitivity to organic Ca^{2+} antagonist drugs including dihydropyridines, phenylalkylamines and benzothiazepines. *T*-type currents, on the other hand, are transient currents that activate at low voltages, inactivate rapidly, deactivate slowly, have small single-channel conductance, and are insensitive to both Ca^{2+} antagonist drugs and polypeptide toxins from snail and spider venoms. Like *L*-type currents, *N*-type, *P/Q*-type, and *R*-type currents also require strong depolarization for activation. They are, however, relatively insensitive to organic Ca^{2+} antagonist drugs, but are defined by specific snail and spider toxins that block them. Based on the voltage-activation threshold, these five channel types can be divided into two classes: high voltage-activated channels (HVA) and low voltage-activated channels (LVA). Sometimes a third group, intermediate voltage-activated (IVA) channels, is also mentioned (Yamakage and Namiki 2002).

4.2 Molecular composition

HVA calcium channels are hetero-oligomeric protein complexes composed of three to four subunits, α_1 , $\alpha_2\delta$, β , and in some cases γ . (Figure 3) (Catterall 2000). The α_1 -subunit is a multi-spanning membrane protein that forms an aqueous pore to the lipid bilayer (Jurkat-Rott and Lehmann-Horn 2004). The protein is organized into four repeat domains (I-IV) each containing six transmembrane domains (S1-S6). The segments S5 and S6 and the membrane associated loop between them form the pore lining, whereas S4 serves as the voltage sensor

initiating conformational changes that open the channel. In addition to constituting the channel pore, voltage-sensor and gating apparatus, the α_1 -subunit determines the main binding sites for regulatory agents, including second messengers, drugs and toxins (Catterall et al. 2005).

The β -subunit is a cytoplasmic protein that binds to the α_1 -subunit at the α_1 interaction domain (AID) on the I-II linker region (Richards et al. 2004). Multiple interactions between α_1 - and β -subunits are crucial for regulation mediated by the β -subunit, including trafficking of the channel complex to the cell membrane, and modulating the channel gating properties (Maltez et al. 2005).

The $\alpha_2\delta$ -subunit is a heavily glycosylated protein derived from a common precursor by proteolytic cleavage to α_2 - and δ -peptides, which remain linked to each other by disulfide bridges (Davies et al. 2007). The α_2 -subunit is extracellular and interacts with α_1 , whereas δ has a single transmembrane segment, which anchors the protein to the cell membrane (Gurnett et al. 1997). The $\alpha_2\delta$ -subunit acts as a channel modulator by enhancing trafficking of the channel complex to the plasma membrane (Canti et al. 2005), and regulating the biophysical properties of the channel, including activation and inactivation kinetics and voltage-dependence (Felix et al. 1997).

The γ -subunit is a four transmembrane domain glycoprotein, originally detected only in skeletal muscle VDCCs (Jay et al. 1990), but recently indicated to associate also with neuronal *P/Q*- and *N*-type channels (Kang et al. 2001). The main function of the γ -subunit is to modulate

the biophysical properties of the channel, but unlike other accessory subunits, the γ -subunit does not seem to have a significant role in trafficking of the channel complex to the cell membrane (Arikkath et al. 2003). In addition to modulation of HVA calcium currents in skeletal muscle and neurons, the γ -subunit may have other biological functions, such as regulation of α -amino-3-hydroxyl-5-methyl-4-isoxazolepropionate (AMPA) receptor membrane targeting (Chen et al. 2000). The molecular composition of the LVA calcium channel accessory subunits is less clear.

4.3 Calcium channel genes

The functional diversity of VDCCs is generated

both by multiple genes and by alternative splicing of RNA transcripts. Mammalian α_1 -subunits are encoded by at least ten distinct *CACNA1* genes (Table 2). Amino acid sequence comparisons of protein products of the α_1 -subunit genes define three subfamilies, Ca_v1 , Ca_v2 , and Ca_v3 , with intra-family sequence identities above 70% (Catterall et al. 2005). Calcium channel α_1 -subunit diversity is enhanced by alternative splicing of pre-mRNA. Genes for α_1 -subunits are well conserved at coding regions for transmembrane segments S1-S5, but the remaining regions are less conserved and show variable degrees of alternative splicing (Jurkat-Rott and Lehmann-Horn 2004). The diversity of Ca^{2+} channel structure and function is further enhanced by multiple accessory subunit genes.

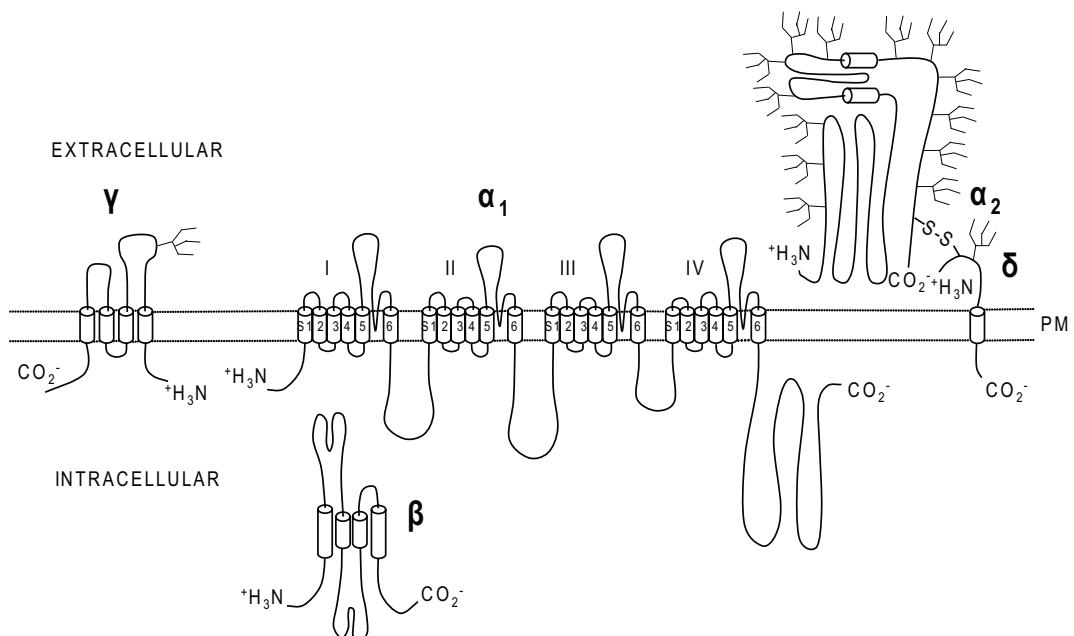


Figure 3. Molecular composition of VDCC purified from skeletal muscle: a pore-forming α_1 -subunit of 190 kDa is associated with a disulphide-linked $\alpha_2\delta$ -dimer of 170 kDa, an intracellular β -subunit of 55 kDa, and a transmembrane γ -subunit of 33 kDa. Cylinders indicate alpha-helices and branched lines in $\alpha_2\delta$ - and γ -subunits represent sugar units. PM, plasma membrane. Modified from Catterall et al. (2000).

Four distinct β -subunit genes, *CACNB1-CACNB4*, have been identified (Birnbaumer et al. 1998). Amino acid sequence comparisons between the β -subunits reveal five domains D1-D5 (Birnbaumer et al. 1998). Two of the domains (D2 and D4) exhibit high homology among the four β -subunits, and resemble the SH3 fold (D2) and guanylate kinase fold (D4) of membrane-associated guanylate kinase (MAGUK) proteins (McGee et al. 2004). The amino terminus (D1), small central linker (D3), and carboxy terminus (D5), are less conserved and undergo extensive alternative splicing (Foell et al. 2004). Recently, new short splice variants were described, which lack large regions of the central conserved domains but still yield functional channel complexes (Harry et al. 2004). At least four distinct genes, *CACNA2D1-CACNA2D4*, encode the α_2 -subunits (Qin et al. 2002). Alternative splicing have been described for *CACNA2D1* (Angelotti and Hofmann 1996), *CACNA2D2* (Hobom et al. 2000), and *CACNA2D4* (Qin et al. 2002). The γ -subunit gene family consists of eight members, *CACNG1-CACNG8* (Kang and Campbell 2003). The γ -subunit is the least studied calcium channel subunit, and alternative splicing has reported only for *CACNG6* (Burgess et al. 2001; Chu et al. 2001).

Many cells may express several subtypes of distinct subunits, and for example, distinct β - and $\alpha_2\delta$ -subunits can interact promiscuously with α_1 -subunits (Dalton et al. 2005; Klugbauer et al. 2003). Functionally diverse calcium channels are presumably formed from different combinations of α_1 -, $\alpha_2\delta$ -, β -, and γ -subunits, but the physiological role of the different combinations is still unclear (Qin et al. 2002).

4.4 Calcium channelopathies

Mutations in the genes encoding VDCC subunits in humans result in a variety of inherited diseases or "channelopathies". Mutations in the *CACNA1A* gene encoding the α_1 -subunit of neuronal P/Q-type calcium channel $\text{Ca}_v2.1$, cause familial hemiplegic migraine type 1 (FHM1), episodic ataxia type 2 (EA2), and spinocerebellar ataxia type 6 (SCA6) (Ophoff et al. 1996; Zhuchenko et al. 1997). Mutations in the *CACNA1C* gene encoding the L-type $\text{Ca}_v1.2$ channel α_1 -subunit are associated with Timothy syndrome (long QT syndrome 8), which is characterized by multiorgan dysfunction, including lethal arrhythmias, webbing of fingers and toes, congenital heart disease, immune deficiency, intermittent hypoglycemia, cognitive abnormalities, and autism (Splawski et al. 2004), or Brugada syndrome (short QT syndrome) causing sudden cardiac death (Antzelevitch et al. 2007). *CACNA1S* mutations of the skeletal muscle L-type $\text{Ca}_v1.1$ α_1 -subunit may result in hypokalemic periodic paralysis (HPP1) characterized by hypotonia and muscle weakness (Ptacek et al. 1994) or malignant hyperthermia susceptibility (MHS) triggered by anesthetics or depolarizing muscle relaxants (Monnier et al. 1997). Mutations of the T-type $\text{Ca}_v3.2$ channel α_1 -subunit encoded by *CACNA1H* are implicated in childhood absence epilepsy (Chen et al. 2003), whereas a variant of the R-type $\text{Ca}_v2.3$ channel α_1 -subunit is suggested to associate with type 2 diabetes (Li Muller et al. 2007). VDCCs are upregulated in many cancer cells, and their role in the progression of cancer (cell proliferation, differentiation and motility) is currently under active investigation (Fiske et al. 2006). Mutations in the auxiliary subunits may

lead to similar phenotypes as those caused by α_1 -subunit mutations; *CACNB4* encoding β_4 -subunit is associated with epilepsy and ataxia (Escayg et al. 2000), whereas mutations of *CACNB2* encoding the β_2 -subunit are involved in Brugada syndrome (Antzelevitch et al. 2007).

VDCCs also have a role in the development of several retinal diseases. As mentioned earlier, mutations in *CACNA1F* encoding the retinal L-type $\text{Ca}_v1.4$ α_1 -subunit lead to X-linked recessive retinal disease, CSNB2 (Bech-Hansen et al. 1998; Strom et al. 1998). In addition to CSNB2, several other retinal diseases are shown to associate with *CACNA1F* mutations, including AIED-like phenotype (Wutz et al. 2002), retinal and optic disc atrophy with progressive decline of visual function (Nakamura et al. 2003), and severe CSNB2-like phenotype associated with female carrier symptoms and intellectual disability (Hope et al. 2005). Recently, mutations in one of the auxiliary subunits, $\alpha_2\delta_4$ encoded by *CACNA2D4*, were also shown to lead to a retinal phenotype in a family with autosomal recessive cone dystrophy (Wycisk et al. 2006).

A number of spontaneous or engineered mutations in mouse VDCC subunit genes have been related with many neuronal, muscular, and retinal phenotypes in mice (Felix 2006). Two different *CACNA1F* mutant mice exist, both displaying retinal dysfunction similar to CSNB2 in humans (Chang et al. 2006; Mansergh et al. 2005). β_2 -subunit null mice expressing the rat β_2 -subunit in cardiac muscle also display a retinal phenotype similar to CSNB2 (Ball et al. 2002). No human β_2 -subunit mutations have, however, been described in CSNB patients. Complete β_2 -subunit gene

null mutations are lethal in mice because of lack of cardiac contraction (Ball et al. 2002), which is consistent with the finding that human β_2 -subunit mutations are reported to cause sudden cardiac death (Antzelevitch et al. 2007). Information on possible retinal dysfunction in cardiac patients carrying β_2 -subunit mutation is unavailable. The discovery and careful analysis of mouse models will help to clarify the role of different mutations in calcium channel function and the pathogenesis of associated diseases (Felix 2006).

AIMS OF THE STUDY

Prior to this thesis work, two X-linked cone-rod dystrophy loci had been localized, *CORDX1* to Xp21.1-11.4 (Bergen et al. 1993; Hong et al. 1994; Seymour et al. 1998) and *CORDX2* to Xq27-28 (Bergen and Pinckers 1997). The disease gene for Åland Island eye disease had been localized to Xp11.3-q13.1 (Alitalo et al. 1991; Alitalo et al. unpublished linkage data). During the course of the study, the *RPGR* gene was identified as a causative gene for *CORDX1* (Demirci et al. 2002; Yang et al. 2002). This study was conducted to clarify the genetic background of X-linked retinal diseases. The specific objectives of this study were to determine:

1. the X-chromosomal location of the cone-rod dystrophy locus (*CORDX3*) in a Finnish family
2. the genes responsible for *CORDX3* and Åland Island eye disease
3. mutations in the newly-identified disease gene (*CACNA1F*) in other patients with related retinal phenotypes
4. the functional consequences of the newly-identified disease gene (*CACNA1F*) mutations

MATERIAL AND METHODS

1 Subjects and samples

1.2 Consent and ethics committee permissions

The patients and family members were informed about the aims of these studies. All participants signed an informed consent form before blood samples were collected. Appropriate research permissions were obtained from the Ethics committees of the participating hospitals.

1.3 Cone-rod dystrophy (CORDX) patients

Members of a large six-generation Finnish family with X-linked cone-rod dystrophy (CORDX) were included in linkage (Study I) and mutation (Study II) analyses. The detailed clinical findings of this family have been published earlier by Mäntyjärvi and coworkers (2001). Briefly, in most of the patients (8/10) of this family the onset of the disease was in childhood, whereas some (2/10) developed the disease clearly in adulthood. All affected males showed decreased visual acuity, myopic refraction, red or red-green color vision defects, elevated or missing cone thresholds and elevated rod thresholds in dark adaptation, and affected cone or cone-rod responses in ERG. Visual fields were normal or concentrically constricted. Some patients had also central scotomas or central reduced sensitivity in the visual field. None of the patients had bull's eye lesions in their eye fundi, but myopic changes or irregular pigmentation in the macular area was present. The 12-14 year follow-up study of four patients revealed only slow progression of the disease including refractive changes,

and mild deterioration in visual acuity, color vision, and visual fields. Female carriers of the family were clinically normal. A total of 50 family members, including 7 affected males, provided blood samples for genetic studies. The Family Federation of Finland and the Department of Ophthalmology, University of Kuopio, organized the collection of the samples.

1.4 Åland Island eye disease (AIED) patients

Members of the original seven-generation AIED family were included in Study III. The disease gene of the family had been mapped earlier to Xp11.3-q13.1 between markers MAOA and DXS559 (Alitalo et al. 1991; Alitalo et al. unpublished linkage data). DNA samples from 29 family members with 6 affected males were included in the mutation analysis. The phenotype of the affected males is described in Forsius and Eriksson (1964), Waardenburg (1970), van Vliet and coworkers (1973), and Carlson and coworkers (1991). Briefly, affected males had hypopigmented fundi, decreased visual acuity due to foveal hypoplasia, latent nystagmus, astigmatism, red color vision defects, and progressive axial myopia. Dark adaptation curves were subnormal with elevated thresholds. In ERG, both rod and cone mediated responses were affected. Female carriers did not show any features of the disease, except for slight latent nystagmus in some cases.

1.5 CSNB2 patient

During the course of the study DNA samples from members (an affected male and his mother) of another Finnish family were included in mutation analysis (unpublished experiments

[U]). The patient was diagnosed with CSNB at the age of 9. He had congenital nystagmus, severe photophobia, progressive myopia, astigmatism, and his best corrected visual acuity was reduced, 0.1–0.2 in both eyes (-6.0 cyl +1.5 ax 110/-6.0 cyl +1.5 ax 70). His optic discs were pale, and the ocular fundi were hypopigmented, however, there was normal or slight hyperpigmentation in the maculas, and the foveas seemed normal. The dark adaptation was delayed with missing cone threshold and elevated rod threshold, and the ERG was negative. Due to photophobia and nystagmus, the quality of the ERG was not good enough to distinguish between the complete or incomplete types of CSNB. As the patient had some clinical features in common with the phenotype of the Finnish CORDX family, the disease gene of the Finnish CORDX family was a likely candidate for the defective gene in this family. In the following sections this family is referred to as the CSNB2 family.

1.6 Control samples

DNA samples from 121 Finnish male blood donors and 50 unrelated, healthy Canadian females, and RNA samples extracted from nine lymphoblastoid cell lines of unrelated, healthy Finnish males and females served as controls.

1.7 Cell lines

Epstein-Barr virus-transformed lymphoblastoid cell cultures from two patients of the CORDX family, the patient of the CSNB2 family, a patient of the AIED-family, and from nine unrelated controls were used in splicing studies (II, III, U). Activated peripheral blood T-lymphocytes from a patient and a carrier of the

CORDX family, and from an unrelated control were utilized in splicing experiments as well (II). Commercially available African green monkey kidney cells (COS-1), baby hamster kidney cells (BHK-21), and human embryonic kidney cells (HEK-293) from the American Type Culture Collection (ATCC) were used in the functional experiments (U).

2 Methods

2.1 DNA and RNA extraction (I, II, III, U)

Genomic DNA from peripheral blood leucocytes was extracted by a standard nonenzymatic method (Lahiri and Nurnberger 1991) (I) or with the Gentra Puregene Blood Kit (Qiagen GmbH, Hilden, Germany) (U). RNA was extracted from cultured lymphoblastoid cells with the RNeasy Mini Kit (Qiagen) (II, III) or the RNeasy -4PCR Kit (Ambion Inc., Austin TX, USA) (U), and from activated peripheral blood T-lymphocytes with Trizol reagent (Invitrogen, Burlington, ON, USA) (II). Isolated RNA was treated with DNase (MessageClean Kit, GeneHunter, Nashville, TN, USA or from the RNeasy -4PCR Kit, Ambion Inc.) to remove contaminating DNA, or used directly as a template for first strand cDNA synthesis.

2.2 Polymorphic markers (I)

A total of 39 polymorphic markers, utilized in linkage analysis (I), covered the region Xp22.32-Xq28 (See Results, Figure 4). Two of these markers were SNPs located in *RPGR* gene introns (rs3888228 and SNP1), and 37 were microsatellite markers, mostly dinucleotide repeats. Two of the repeats, CA20 and CA23, were novel markers identified from the National

Center for Biotechnology Information (NCBI, www.ncbi.nlm.nih.gov) database sequence (AC006121). Information on DXS10042 was obtained from Dr. Torben Bech-Hansen, and the other 34 known markers were from the NCBI UniSTS database.

2.3 Gel electrophoresis (PAGE, SSCP) (I)

PCR-amplified alleles of the microsatellite markers were fractionated in 6% polyacrylamide (PAGE) gels (National Diagnostics, Atlanta, GA, USA). Single-strand conformation polymorphism (SSCP) gel electrophoresis was applied to separate the *RPGR* gene SNP alleles used in linkage analysis, and to screen the candidate genes (*DDX3*, *NYX*, *GPR34*, *I-4*, *NDP*, *RP2*, *TIMP1*, and *ARR3*) for mutations. Exons and the genomic regions containing SNPs were PCR-amplified from genomic DNA, after which the PCR products were separated on 0.5-1.0 x MDE gels (BioWhittaker Molecular Applications, Rockland, ME, USA). PAGE- and SSCP-gels were visualized by silver staining (Bassam et al. 1991).

2.4 Linkage and haplotype analysis (I)

For linkage and haplotype analyses, 50 family members of the CORDX family were genotyped with the 39 X-chromosomal markers. Haplotypes were constructed manually. Linkage analysis was carried out using the MLINK program of the FastLink package, version 4.1P (Cottingham et al. 1993). The inheritance mode was set to X-linked with full penetrance, assuming disease allele frequency of 0.00001. Allele frequencies for the markers were calculated from the unaffected family members.

2.5 Sequencing (I, II, III, U)

Candidate genes (*DDX3*, *NYX*, *GPR34*, *I-4*, *NDP*, *RP2*, *TIMP1*, *ARR3*, *RPGR* [I], and *CACNA1F* [II, III, U]) were screened for mutations by direct DNA sequencing. Exons and exon-intron boundaries of the genes were PCR-amplified from genomic DNA. Primers were designed with the Primer 3 program (<http://frodo.wi.mit.edu/primer3/input.htm>). For the *CACNA1F* gene, published primer sequences were used (Boycott et al. 2001). PCR-products were purified either with the QIAquick PCR Purification Kit (Qiagen), or with ExoSAP-IT (USB, Cleveland, OH, USA), and sequenced with the ABI PRISM BigDye Terminator v3.0/v3.1 Cycle Sequencing Kit and the ABI310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

2.6 mRNA/cDNA analysis (II, III, U)

Reverse transcriptase polymerase chain reaction (RT-PCR) was used to assess the naturally occurring *CACNA1F* mRNA splice variants, and aberrant variants caused by *CACNA1F* mutations. Human retinal cDNA (Human Retina QUICK-Clone cDNA library; BD-Clontech, Palo Alto, CA, USA), and lymphoblastoid/T-cell cDNA, synthesized with M-MLV (H-) enzyme and random hexamers (Promega, Madison, WI, USA) (II, III, U), or with Omniscript RT Kit (Qiagen) (II), were used as templates in PCR with *CACNA1F* exonic primers. Primers for cDNA studies were designed with the Primer 3 program.

The regions studied from *CACNA1F* cDNA were the following: the sequence from exons 27-28 to exons 33-34 was studied from

retinal cDNA, cDNA from activated T-cells of a CORDX patient, a carrier female and an unrelated control, and from lymphoblastoid cDNA of two CORDX patients, an AIED patient, and nine unrelated controls (II, III). The exon 14-18 region was studied from the patient of the CSNB2 family and an unrelated control (U). The region of interest was PCR-amplified from the cDNA. Three approaches were then applied to separate the different sized PCR-products. First, PCR-fragments were separated on 1-2% Metaphor agarose gels (BioWhittaker Molecular Applications), excised, and column purified (Qiagen). Second, PCR-products were fractionated on 6% PAGE gels, excised, reamplified, and column purified (Qiagen). Third, PCR-products were cloned into the pCR2.1-TOPO cloning vector (Invitrogen, Carlsbad, CA, USA), and plasmids were extracted with the Plasmid Mini Kit (Qiagen). Purified DNA-fragments and plasmids were then sequenced with the ABI310 Genetic Analyzer (Applied Biosystems).

2.7 Restriction fragment length polymorphism (RFLP) (II, U)

RFLP analysis was utilized to screen the *CACNA1F* mutations, IVS28-1 GCGTC>TGG (II) and IVS16+2T>C (U), among the family members and control samples. The region of interest was PCR amplified and then digested either with restriction endonuclease (New England Biolabs, Beverly, MA, USA) *Fnu4HI* (IVS28-1 GCGTC>TGG) or with *HpyCH4III* (IVS16+2T>C). Digested PCR-products were run on 2% agarose gels and visualized with ethidium bromide under UV light.

2.8 *In vitro* expression of the $\text{Ca}_v1.4$ calcium channel (U)

Subcellular localization of the $\text{Ca}_v1.4$ channel was studied by transient transfections (U). Expression vectors, α_1 -pCIneo, α_1 -pGFP (GFP, green fluorescent protein), β_3 -pCMV, and $\alpha_2\delta_1$ -pCDNA3, representing the $\text{Ca}_v1.4$ calcium channel subunits, were kindly provided by Professor Jörg Striessnig (Hoda et al. 2005; Koschak et al. 2003). Mouse clarin-1 cDNA cloned in pEGFP-N1 expression vector (Clontech) served as a transfection and plasmamembrane localization control. For transient transfections, BHK-21, COS-1 or HEK-293 cells were plated on 15 mm coverslips in 6-well plates 20 hrs before transfection with FuGENE 6 (Roche, Indianapolis, IN, USA) or Lipofectamine (Invitrogen) transfection reagent. The human $\text{Ca}_v1.4$ α_1 cDNA clone was transfected alone (1.5 μg), or cotransfected together with the auxiliary subunits, β_3 and $\alpha_2\delta_1$, in equimolar concentrations. After transfections, cells were incubated either at 37°C for 12-72 hrs, at 30°C for 12-48 hrs, or first at 37°C for 6 hrs and then at 30°C for the next 12-48 hrs.

2.9 Immunofluorescence staining of transfected cells (U)

The α_{1F} proteins in transfected cells were visualized with a GFP-tag or indirect immunofluorescence. Cells growing on coverslips were fixed with 4% paraformaldehyde in phosphate buffered saline (PBS), permeabilized with 0.2% saponin and then blocked with 2-5% bovine serum albumin or 10% nonimmune donkey serum (Jackson ImmunoResearch, West Grove, PA, USA). Polyclonal antibody,

produced in sheep against the human $\text{Ca}_v1.4$ α_{1F} SNEKDLPQENEGLVPGVEK peptide, was kindly provided by Dr. Catherine Morgans (Morgans 2001). Secondary donkey anti-sheep antibodies were either Cy2 (Jackson ImmunoResearch) or Alexa546 (Invitrogen) conjugated. Stained cells were viewed and documented with a Zeiss Axioplan 2 microscope and digital camera, and Axiovision 3.1 software.

RESULTS AND DISCUSSION

1 Identification of a third X-linked cone-rod dystrophy locus, *CORDX3* (I)

1.1 Linkage and haplotype analyses

In order to localize the disease gene for cone-rod dystrophy (CORDX, formerly COD) in the Finnish family (Mäntyjärvi et al. 2001), we performed linkage and haplotype analyses with markers from Xp22.32 to Xq28, covering both of the previously identified loci, *CORDX1* (formerly *COD1*) in Xp21.1-11.4 (Bergen et al. 1993; Hong et al. 1994; Seymour et al. 1998) and *CORDX2* (formerly *COD2*) in Xq27-28 (Bergen and Pinckers 1997). At the initial stage of our mapping studies, the disease gene for CORDX1 was still unidentified, and the critical regions for CORDX1 and CORDX of the Finnish family were partially overlapping for approximately 300 kb in the Xp11.4 region (Figure 4). After the causative gene, *RPGR*, for CORDX1 was identified (Demirci et al. 2002; Yang et al. 2002), recombination events between the disease gene of the Finnish family and markers in the *CORDX1* region, including an *RPGR* intragenic SNP1, resulted in exclusion of the *RPGR* gene as the causative gene in the Finnish CORDX family. Sequencing of the *RPGR* gene verified the exclusion. Lod scores for all the markers in the Xq27-28 region were negative, also excluding the *CORDX2* region as the site of the disease gene in the Finnish CORDX family. We were thus able to identify a new CORDX locus, *CORDX3* (I, MIM 300476).

Two-point linkage analysis showed linkage without recombination between CORDX3 and ten markers from the pericentromeric region of

the X-chromosome (Figure 4). Lod scores were significant for four of these markers, DXS993 ($Z_{\max}=3.90$ at $\theta=0.00$), MAOB ($Z_{\max}=3.48$ at $\theta=0.00$), and DXS1055 ($Z_{\max}=3.18$ at $\theta=0.00$) from the Xp region, and DXS1194 ($Z_{\max}=4.10$ at $\theta=0.00$) from the Xq region. DXS10042 in the Xp11.4 region and DXS8060 in Xq13.1 were the closest flanking markers showing recombinations with respect to the disease gene. The genetic distance between these markers was approximately 35 cM (Figure 4). Genetic heterogeneity among cone-rod dystrophies (see Table 1), and among inherited retinal diseases in general, is a well-established phenomenon. Therefore, identification of a new CORDX locus was not surprising, but further expanded the knowledge concerning the genetic background of cone-rod dystrophies.

1.2 Candidate gene screening from the *CORDX3* region

A total of nine candidate genes were screened for mutations from the *CORDX3* region (I, II) (Figure 4). From the 300 kb region that was initially shared by *CORDX1* and *CORDX3*, four genes, *DDX3*, *NYX*, *GPR34*, and *I-4* were screened, but no mutations were identified (I). After identifying *CORDX3* as a distinct locus, we continued candidate gene screening by choosing functionally attractive genes for mutation analysis from the *CORDX3* region. Disease genes for other inherited retinal diseases, including *NDP* responsible for Norrie disease (Berger et al. 1992) and *RP2* causing retinitis pigmentosa (Schwahn et al. 1998), were chosen because of the well-known allelic heterogeneity among inherited retinal diseases. *TIMP1* was an attractive candidate gene because of its involvement in modification

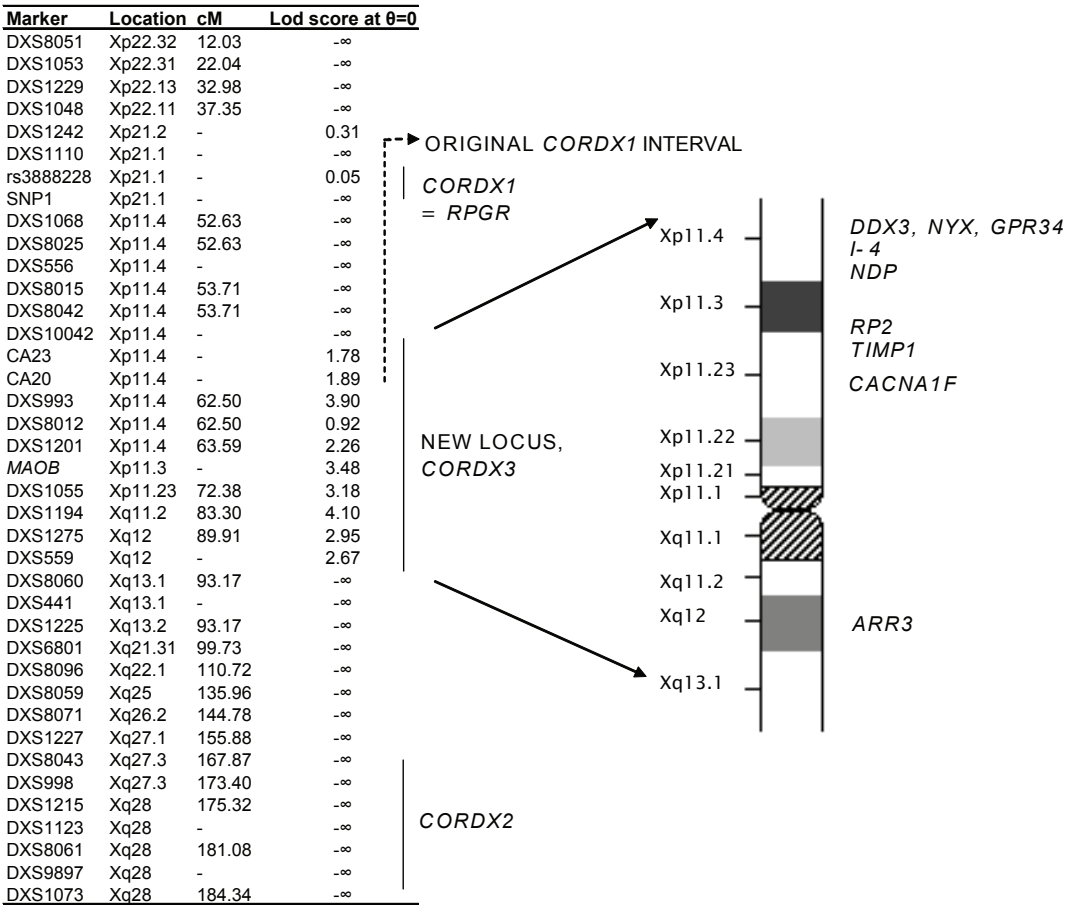


Figure 4. Two-point linkage between the disease gene of the Finnish CORDX family and X-chromosomal markers, the locations of the X-linked cone-rod dystrophy loci (CORDX), and the candidate genes chosen for mutation analysis.

of the interphotoreceptor matrix (Plantner et al. 1998). Also, mutations in *TIMP3*, which belongs to the same gene family as *TIMP1*, were known to cause Sorsby's fundus dystrophy (Weber et al. 1994). *ARR3*, on the other hand, was screened because of its retina-specificity and involvement in retinal signal transduction (Murakami et al. 1993). Screening of these four new candidate genes revealed no mutations (I). All the nucleotide changes observed were polymorphisms present also in subsets of the 100 control samples studied.

2 The calcium channel α_1 -subunit gene, *CACNA1F*, as a candidate gene in retinal diseases (II, III, U)

2.1 *CACNA1F* gene structure and function

The L-type calcium channel α_{1F} -subunit gene, *CACNA1F*, is a functional candidate gene for many retinal diseases mapping to the same genetic interval as *CACNA1F*, including diseases studied in this thesis. The *CACNA1F* gene consists of 48 exons spanning 28 kb in

the Xp11.23 region (Bech-Hansen et al. 1998; Strom et al. 1998). The gene was first identified as a causative gene for an incomplete type of congenital stationary night blindness, CSNB2 (Bech-Hansen et al. 1998; Strom et al. 1998). Subsequent studies indicated mutations also in patients with related retinal phenotypes, including CSNB2 with atypical retinal atrophy and visual field defects (Nakamura et al. 2002), retinal and optic disc atrophy with progressive decline of visual function (Nakamura et al. 2003), severe CSNB2-like phenotype with intellectual disability and symptoms in female carriers (Hope et al. 2005), and AIED-like phenotype (Wutz et al. 2002).

The *CACNA1F* gene codes for the α_{1F} -subunit of the L-type $\text{Ca}_v1.4$ calcium channel. In the rat and mouse retina, the channel is located in both the outer plexiform layer (OPL) and in lesser amounts in the inner plexiform layer (IPL) (Berntson et al. 2003; Morgans 2001; Morgans et al. 2001; Morgans et al. 2005). In the OPL, the channel is colocalized with rod and possibly cone photoreceptor synaptic ribbons, which are part of the active zone complex involved in glutamate release from photoreceptor synaptic terminals. In macaque retina, with more cones than rodent retina, the channel is clearly present in both the rod and the cone terminals (Wässle et al. 2003). The expression profile of *CACNA1F* is retina-enriched, but *CACNA1F* mRNA and/or α_{1F} -protein is also present in adrenal gland, spleen, spinal cord, bone marrow, thymus, skeletal muscle (McRory et al. 2004), pineal gland (Hemara-Wahanui et al. 2005), and T-cells (Kotturi et al. 2003), suggesting a wider role in human physiology. Many of the tissues expressing *CACNA1F* are associated with the immune system, however, potential defects of

immune response in patients with *CACNA1F* mutations have not been reported.

2.2 *CACNA1F* mutations in Finnish patients

2.2.1 Mutation of *CACNA1F* leads to CORDX3 (II)

The *CACNA1F* gene was an attractive candidate gene for CORDX3, because of its involvement in retinal signaling and in the pathogenesis of several retinal diseases. Sequencing of the *CACNA1F* gene from CORDX3 patients revealed a novel deletion/insertion mutation, IVS28-1 GCGTC>TGG, in the splice acceptor site of intron 28 (II). This mutation co-segregated with the disease phenotype in the CORDX3 family and was not observed in the 200 control chromosomes studied.

As the identified mutation affected a splice site of the *CACNA1F* gene, we decided to study the effect of this mutation on pre-mRNA splicing (see section 2.3). Naturally, we could not study *CACNA1F* pre-mRNA splicing in patients' retina, which is the main site of *CACNA1F* expression. We therefore studied the expression of *CACNA1F* mRNA in normal controls in tissues that would also be easily obtainable from the patients, such as fibroblast and lymphoblastoid cells. RT-PCR experiments indicated that *CACNA1F* mRNA was expressed, although at relatively low levels, in cultured lymphoblastoid cells (II).

2.2.2 Finding *CACNA1F* mutations in AIED (III)

The *CACNA1F* gene has long been a positional as well as a functional candidate gene also for AIED. Even though mutations were found from AIED-like patients, surprisingly, a previous sequencing study identified no mutations in the genomic DNA of patients belonging to the original AIED family (Wutz et al. 2002). Because the *CACNA1F* gene expression was originally reported to be restricted to the retina (Bech-Hansen et al. 1998; Strom et al. 1998), and retinal RNA from AIED patients for mutation studies was unavailable, mutations in intronic or regulatory regions could not be ruled out. Our studies of the CORDX3 family indicated *CACNA1F* expression also in lymphoblastoid cells (II), which were easily available from AIED patients, and thus enabled the screening the *CACNA1F* gene from both genomic DNA and mRNA.

Our initial sequencing of the *CACNA1F* gene from genomic DNA of an AIED patient revealed no mutations either. We, however, failed to amplify exon 30 robustly with published primers, while other exons amplified properly. To amplify and sequence exon 30, we then used new primers from the nearby exons. Amplification of exons 27 to 31 revealed a novel 425 base pair (bp) genomic deletion, covering part of intron 29 (133bp), the whole exon 30 (111bp) and part of intron 30 (181bp) (III). Sequencing of *CACNA1F* mRNA from patient lymphoblastoid cells confirmed the skipping of exon 30. The mutation was present in all affected males and female carriers of the AIED family, and it was absent from unaffected family members and the 121 control chromosomes studied.

2.2.3 *CACNA1F* mutation in a Finnish *CSNB2* patient (U)

The *CACNA1F* gene was sequenced from a Finnish *CSNB2* patient based on the observation that the patient showed clinical features of both *CSNB2*, and *CORDX3*. A novel mutation, IVS16+2T>C, in the splice donor site of intron 16 was identified (U). The mutation was present in the patient and his carrier mother, but it was absent from the 100 control chromosomes studied.

2.3 Alternative splicing of the *CACNA1F* gene

2.3.1 *CACNA1F* mutations affect pre-mRNA splicing (II, III, U)

As the three newly-identified mutations, IVS16+2T>C (U), IVS28-1 GCGTC>TGG (II), and 425 bp genomic deletion of exon 30 and adjacent intronic regions (III), affected splice sites of the *CACNA1F* gene, we carried out *CACNA1F* mRNA analysis from exon 14 to 18 and from exons 27-28 to 33-34 flanking the mutation sites. Analysis of *CACNA1F* mRNA from lymphoblastoid cells or activated T-cells of patients and controls revealed aberrant splicing products due to mutations as well as novel alternatively spliced wild-type (wt) *CACNA1F* variants (II, III, U, Figure 5A).

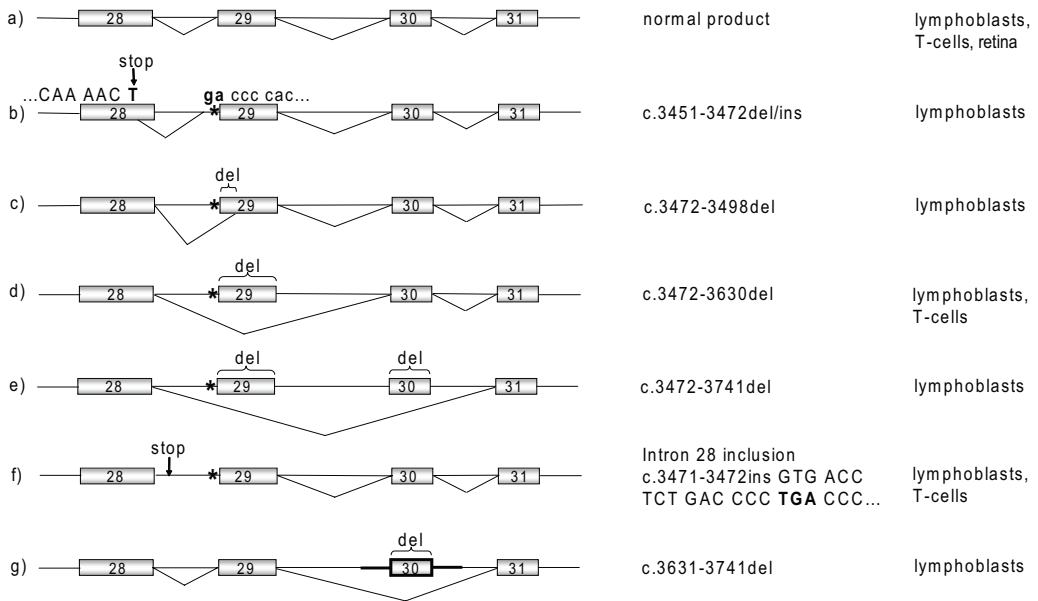
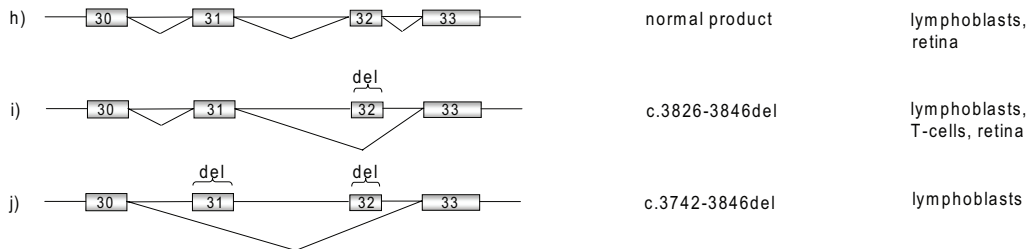
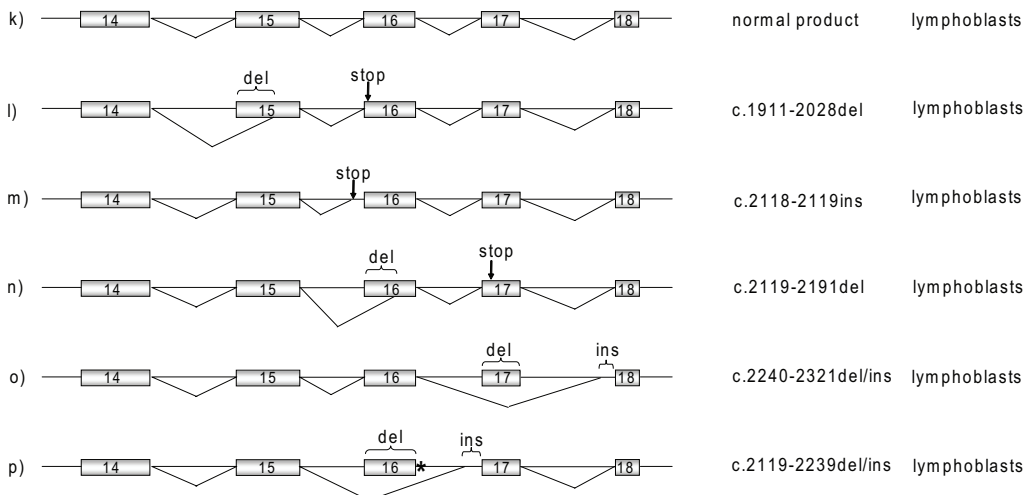
The analysis of the *CACNA1F* exon 28-30 region from the lymphoblastoid-derived RNA of *CORDX3* patients with the IVS28-1 GCGTC>TGG mutation revealed five aberrantly spliced variants (II, Figure 5A, b-f). Control samples showed only wt transcript. The analysis of RNA further upstream of the mutation site

revealed two additional splice variants: skipping of exon 32 and skipping of exons 31-32 (Figure 5A, i-j). These variants were present also in all control samples studied (II, III). Analysis of RNA of activated T-cells from a CORDX3 patient and carrier female revealed only two of the aberrant splice variants (II, Figure 5A, d and f). Skipping of exon 32 was present in both patient and control T-cells, whereas skipping of exons 31-32 was undetectable in T-cells. All variants could not be sequenced from activated T-cells due to RT-PCR favoring major variants over low-expressed variants. There is thus a possibility that the CORDX3 mutation may lead to formation of yet additional aberrant splicing products with very low expression level. The five aberrant splice variants observed predict different sized in-frame deletions from the III-IV linker region to repeat domain IV, or premature termination codons (PTC) in the linker between repeat domains III and IV of the $\text{Ca}_v1.4$ channel (Figure 5B, b-f). The variants with PTCs are most likely destroyed by nonsense-mediated mRNA decay (NMD), a pathway that degrades mRNAs that have PTCs in their open reading frames >50 nucleotides upstream of the last exon-exon junction to prevent accumulation of potentially toxic polypeptide fragments (Baker and Parker 2004). This suggestion is supported by a recent transient expression study, which indicated that PTC in the intracellular C-terminus of $\text{Ca}_v1.4$ seems to lead to the absence of the protein (Hoda et al. 2005). Quantitative RT-PCR analysis, however, is needed to confirm the actual levels of mRNA variants with PTCs and the probable involvement of NMD. Variants which predict in-frame deletions in repeat domain IV may produce a protein. Two of these variants remove IVS1 or IVS1-S2 transmembrane segments, and the third

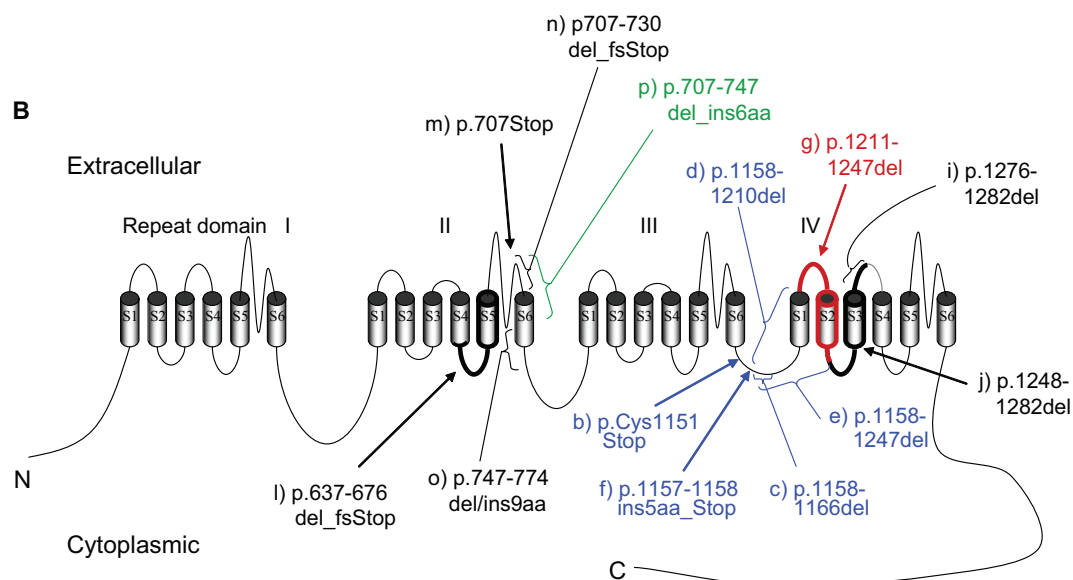
predicts a small deletion of the III-IV linker region. Removal of the IVS1 transmembrane segment, furthermore, predicts altered membrane topology for the C-terminal part of the protein. Functional consequences of these deletions on the $\text{Ca}_v1.4$ channel are unknown, however, even missense mutations are known to cause marked changes in channel gating, or completely prevent the channel from functioning (Hoda et al. 2005; Peloquin et al. 2007). Based on these observations it is highly likely that the mutation in CORDX3 patients results in reduced levels of the $\text{Ca}_v1.4$ channel with altered or absent channel function.

In AIED, which is caused by the 425 bp genomic deletion of *CACNA1F* exon 30 and flanking intronic regions, analysis of lymphoblastoid RNA revealed an aberrant splicing product which lacked exon 30 (III, Figure 5A, g). The variants lacking exon 32 and exons 31-32, observed in CORDX3 patients and control samples, were also observed in the AIED patient. Deletion of exon 30 predicts in-frame deletion of the protein, involving transmembrane domain IVS2 and the preceding extracellular loop. As a consequence, membrane topology of the C-terminal part of the protein is predicted to change. A similar kind of membrane topology change is predicted by one of the aberrant CORDX3 splice variants (Figure 5B, d), as well as the alternative wt *CACNA1F* splice variant in lymphoblastoid cells with skipping of exons 31-32 (Figure 5B, j). Because of the altered membrane topology, these variants are likely to yield nonfunctional channels or significantly altered channel function (see section 2.3.2).

The analysis of the *CACNA1F* exon 14-18 region from lymphoblastoid RNA of the CSNB2

A Exon 28-31 region, consequences of the CORDX3 (b-f) and AIED (g) mutations**cDNA source****Exon 30-33 region, normal variants****Exon 14-18 region, normal variants (l-o) and consequence of the CSNB2 mutation (p)**

< Figure 5A. *CACNA1F* transcripts detected with RT-PCR. Exon 28-31 region: normal splicing product (a), five different outcomes of the mutation, IVS28-1 GCGTC>TGG, in CORDX3 patients (b-f), and outcome of the 425bp deletion in AIED patients (g). Exon 30-33 region: normal splicing product (h), and two alternative splice variants (i-j). Exon 15-17 region: normal splicing product (k), four alternative splice variants (l-o), and outcome of the mutation, IVS16+2T>C, in a CSNB2 patient (p). An asterisk or bold lines denote the locations of the respective mutation sites.



B. Putative membrane topology of the human L-type calcium channel $\text{Ca}_v1.4 \alpha_{1F}$ subunit encoded by *CACNA1F*, and the predicted consequences of detected mutations (in colors) and alternative splice variants (in black) for the protein. The alphabetical order of the variants is similar in 5A and B. Numbering is according to NCBI nucleotide (NM_005183) and protein (NP_005174) sequences.

patient with the mutation IVS16+2T>C revealed an aberrantly spliced variant which was absent from a control sample (U, Figure 5A, p). The control sample showed four alternative splice variants (Figure 5A, l-o) in addition to the wt transcript containing all exons. The mutation-induced aberrant splice variant predicts an in-frame deletion/insertion in the IIS6 segment and the membrane associated loop between IIS5-S6 (Figure 5B, p). The functional consequence of this mutation is unknown, however, it is predicted to result in a nonfunctional channel or markedly altered channel function.

2.3.2 *Alternative splicing enhances channel diversity (II, III, U)*

Splicing, *i.e.* removal of introns from pre-mRNA, is an important step in gene expression. While the estimation of the number of genes in the human genome is getting smaller and smaller, the importance of alternative splicing as a mechanism increasing the functional diversity of proteins is becoming more apparent. In the early 1980's, researchers estimated that 5% of genes in higher eukaryotes undergo alternative splicing, but according to the latest reports at least 35-60% of the genes in the human genome show evidence of alternative splicing (Modrek and Lee 2002). Alternative splicing may affect the protein in a variety of ways, for example by replacing the N- or C-terminus, adding or removing a functional unit, or producing a truncated protein product. Consequently, the binding properties, post-translational modifications, intracellular localization, enzymatic activity, or the stability of the protein may change (Stamm et al. 2005). Alternative splicing is often tissue- or development-specific, and therefore certain

transcript variants may be present only in certain cell types or specific developmental stages. In addition to the traditional view that alternatively spliced transcripts encode alternative proteins, recent data suggests that many genes produce also other transcripts that may include sequences from both strands, neighboring genes, or intragenic regions (Birney et al. 2007; Gingeras 2007). Some of these transcripts, called transcripts of unknown function (TUFs), appear to have a role in the regulation of protein-coding gene expression, and others may have as yet unknown regulatory functions (Gingeras 2007).

For the *CACNA1F* gene, alternative splicing has been described for exons 1, 2, 9 (Boycott et al. 2001), and 32 (McRory et al. 2004) in retina, and for exons 31, 32, 33, 34, and 37 in lymphocytes (Kotturi and Jefferies 2005). Our experiments with lymphoblastoid RNA indicated alternative splicing for *CACNA1F* exons 15, 16, 17, 31, and 32 (Figure 5A, i-j, l-o). Alternative splicing within exons 15, 16, and 17 involved the use of alternative splice acceptor sites (U), whereas exons 31 and 32 were alternative exons either included in the transcript or excluded by exon skipping (II, III). Analysis of a retinal cDNA library demonstrated the skipping of exon 32, while skipping of exons 31-32 did not occur (III).

Exon 32 codes for a short sequence, NGGHLGE, located in the extracellular loop between segments IVS3-S4 of the $\text{Ca}_v1.4$ channel. Wt splice variants involving skipping of a short exon constituting part of this loop have also been described for other L-type calcium channels, $\text{Ca}_v1.1$, $\text{Ca}_v1.2$, and $\text{Ca}_v1.3$ (Barry et al. 1995; Perez-Reyes et al. 1990; Safa et al. 2001; Yu et al. 1992). The changes in this loop

between segments S3-S4 may influence the voltage dependence of the channels because of the close vicinity of the loop to the S4 segment, which is known to act as a voltage sensor initiating conformational changes that open the channel (Bezanilla 2002). For example, $\text{Ca}_v1.2$ channels in heart and brain, which have a shorter IVS3-S4 linker region due to skipping of an exon, show altered voltage-dependent activation (Tang et al. 2004). This may influence the shape or duration of action potentials. Also, N-type $\text{Ca}_v2.2$ and P/Q-type $\text{Ca}_v2.1$ calcium channels with an insertion of two amino acids in the loop between segments IVS3-S4 due to alternative splicing show differences in activation kinetics and voltage-dependence of gating, as well as in sensitivity to spider peptide toxin ω -aga IVA (Bourinet et al. 1999; Lin et al. 1999).

The wt variant which involves skipping of exons 31-32 of the *CACNA1F* gene (Figure 5A, j) was absent from retina, however, it may have a specific function in lymphoblastoid cells (II, III). This variant leads to deletion of the IVS3 transmembrane segment and part of the IVS3-S4 linker region, suggesting an altered membrane topology of the C-terminal part of the protein. Similar splice variants have been described also for the L-type calcium channel $\text{Ca}_v1.3$ in rat neuroendocrine GH3 cells (Safa et al. 2001), and for $\text{Ca}_v1.2$ in human adult heart and fetal brain (Tang et al. 2004). Functional consequences of these variants are unknown. An alternative $\text{Ca}_v1.4$ splice variant in lymphocytes, which involves skipping of exons 31-34 and exon 37, leads to deletion of the IVS3-S5 region and thus probably alters the membrane topology (Kotturi and Jefferies 2005). This variant is hypothesized to form a

functional, but voltage-insensitive, channel. The variants which show skipping of exons 31-32 may also cause changes in channel voltage-sensitivity, or lead to as yet unknown channel function, or a nonfunctional channel.

In the *CACNA1F* exon 14-18 region four alternatively spliced variants were present (U). These variants use alternative splice acceptor sites either in exon 15, intron 15, exon 16, or in intron 17. The variant, which uses an alternative splice acceptor in intron 17 (Figure 5, o), leads to an in-frame deletion/insertion in the IIS6 segment and the following domain II-III linker region. This linker region mediates interactions with effector proteins, and variations in this region may lead to differences in channel function. For example, rat and rabbit splice isoforms of P/Q-type $\text{Ca}_v2.1$ channels with sequence differences in the domain II-III linker region have different patterns of interactions with synaptic proteins (Kim and Catterall 1997). The other three *CACNA1F* variants observed in the exon 14-18 region lead to PTCs in the IIS4-S5 intracellular loop or in the IIS5-S6 extracellular loop (Figure 5, l-n). The production of transcripts with PTCs may serve as a mechanism for gene expression regulation (Stamm et al. 2005). Variants with PTCs are subjected to NMD for degradation, and thus the overall protein expression level is reduced. Approximately 18-25% of all transcripts are down-regulated by NMD. In some cases, transcripts may, however, escape NMD (Bachmann et al. 2006), or transcripts likely to be subjected to NMD may produce small amounts of protein (Dreumont et al. 2005). If the *CACNA1F* variants with PTCs are translated, they produce truncated protein products with only repeat domains I-II present. Several two-domain variants generated by

alternative splicing exist in VDCCs. In rabbit heart alternative splicing removes exons 17-18 or exon 19, leading to expression of an L-type $\text{Ca}_v1.2$ channel consisting only of repeat domains I-II (Wielowieyski et al. 2001). The same channel in human fibroblasts predicts a two-domain isoform resulting from the use of an alternative splice acceptor in exon 15 (Soldatov 1994). A similar two-domain protein variant of the P/Q-type $\text{Ca}_v2.1$ channel is present in synaptic membranes of rabbit brain (Scott et al. 1998). *In vitro* expression of N-type $\text{Ca}_v2.2$ two-domain variants provides clues to the significance of such truncated channel forms (Raghib et al. 2001). When domains I-II or III-IV are expressed individually with auxiliary subunits no functional channels are formed, however, when domains I-II or domains III-IV are coexpressed with full-length $\text{Ca}_v2.2$, they act in a dominant-negative manner suppressing expression of the full-length channel. Based on these data, it seems that the newly-observed $\text{Ca}_v1.4$ variants with PTCs lead to down-regulation of the channel in lymphoblastoid cells, whether translated or not.

Alternative splicing seems to be an important means to produce functional diversity among VDCCs, or down-regulate the expression level of the channel. A number of splice variants have already been described for all VDCCs, however, the total degree of alternative splicing remains still unknown. Systematic scanning of loci for alternative splicing for *CACNA1C* (Tang et al. 2004), and *CACNA1A* (Soong et al. 2002), has revealed extensive alternative splicing, suggesting that splice-related functional diversity of VDCCs may be considerably larger than is currently known. Comprehensive transcript scanning will probably reveal as

yet unidentified splice variants also for the *CACNA1F* gene.

2.4 *CACNA1F* mutation spectrum and associated phenotypes (II, III, U)

To date, a total of 57 *CACNA1F* mutations have been identified (Table 3), including the mutations identified in this thesis. The mutation spectrum is wide, including missense, nonsense, splice site, insertion, and deletion mutations. Mutations are distributed equally along the entire gene, and no mutation hot spots exist. Most *CACNA1F* mutations lead to a CSNB2 phenotype, however, mutations are present also in patients with divergent but overlapping diagnoses, including CSNB2 with atypical retinal atrophy and visual field defects (Nakamura et al. 2002), retinal and optic disc atrophy with progressive decline of visual function (Nakamura et al. 2003), severe CSNB2-like phenotype with female carrier symptoms and intellectual disability (Hope et al. 2005), and an AIED-like phenotype (Wutz et al. 2002). In this thesis, *CACNA1F* mutations were also found in cone-rod dystrophy (CORDX3) patients (II), patients of the original AIED family (III), as well as in a Finnish CSNB2 patient (U).

2.4.1 *Studies of functional consequences of CACNA1F mutations (U)*

Over half of the known *CACNA1F* mutations are nonsense mutations, deletions, insertions, or splice site mutations predicting PTCs (Table 3, mutation numbering according to the NP_005174 protein sequence), and thus are likely to lead to loss-of channel function, which is suggested to be the disease mechanism underlying CSNB2. The functional studies

have supported this hypothesis. Functional expression of C-terminal $\text{Ca}_v1.4$ mutant, W1451X, reveals that a truncated mutant channel does not support ionic currents due to the lack of protein expression (Hoda et al. 2005).

The transient expression studies and patch clamp recordings of the $\text{Ca}_v1.4$ channel in HEK-293 tsA-201 cells or *Xenopus laevis* oocytes bring light also to the disease mechanisms resulting from the *CACNA1F* missense mutations leading to CSNB2. Missense mutations S229P, G1018R, R1060W, and L1079P lead to the absence of channel function although the expression levels are indistinguishable from the wt channel (Hoda et al. 2005; Peloquin et al. 2007), indicating that change of only one amino acid may prevent the channel from functioning completely. On the other hand, missense mutations G369D and F753C do not prevent channel function but cause profound changes in $\text{Ca}_v1.4$ gating properties, suggesting gain-of-function as a disease mechanism in these cases (Hoda et al. 2005; Peloquin et al. 2007). Finally, the mutations R519Q and L1375H suggest nearly normal or unchanged channel properties, but reduce the expression level of the channel (Hoda et al. 2006). Mutants G674D and A928D also suggest unchanged channel properties (McRory et al. 2004), however, the expression levels of these mutants have not been studied, and it is possible that they function in a similar way as R519Q and L1375H by reducing the channel expression (Hoda et al. 2006).

Previous expression studies suggest both loss-of-function and gain-of-function as a disease mechanism underlying the CSNB2

phenotype. Results from the expression studies are not, however, totally in accord with each other. Hoda and coworkers (2005) reported that the W1451X mutant leads to the absence of protein expression. An independent study reported normal current amplitudes and gating kinetics for the same mutant (McRory et al. 2004). These observations may be due to $\text{Ca}_v1.4$ constructs, which represent different splice variants, and in addition have polymorphic amino acid changes in four positions. Additional studies must, however, clarify the role of different splice variants and polymorphisms in $\text{Ca}_v1.4$ channel function.

In this thesis we aimed to investigate the functional consequences of *CACNA1F* mutations leading to CORDX3 and AIED phenotypes by analyzing the subcellular localization of a mutant $\text{Ca}_v1.4$ channel in transiently transfected cells (U). We started the functional studies by expressing the wt $\text{Ca}_v1.4$ in BHK-21, HEK-293, and COS-1 cells. The expression of wt $\text{Ca}_v1.4$ α_{1F} subunit alone resulted in non-plasmamembrane localization of the channel subunit (Figure 6B) presumably due to the absence of auxiliary subunits $\alpha_2\delta$ and β , which are crucial in trafficking the channel to the plasma membrane (Canti et al. 2005; Maltez et al. 2005). The coexpression of wt α_{1F} with the auxiliary subunits $\alpha_2\delta_1$ and β_3 resulted unexpectedly also in non-plasma membrane localization (Figure 6C). We expressed channel subunits in different cell lines and at lower temperatures to achieve lower channel expression levels, which could promote the correct folding and trafficking of the channel to the cell membrane. All our expression experiments, however, resulted in aggregation of the channel protein in the cell, while the

Table 3. Mutations of the *CACNA1F* gene associated with *CSNB2* and related retinal diseases

Exon/intron	Mutation	Type	Predicted consequence on protein product	Reference
2	c.148C>T	nonsense	p.Arg50Stop	Boycott et al. 2001
2	c.151del5bp	deletion	p.Arg51fsStop	Wutz et al. 2002
2	c.220T>C	missense	p.Cys74Arg	Wutz et al. 2002
2	c.244C>T	nonsense	p.Arg82Stop	Boycott et al. 2001, Wutz et al. 2002
4	c.466-469del4bp/ins34bp	deletion/insertion	p.Ser156/del2aa_ins12aa	Nakamura et al. 2001, Boycott et al. 2001, Nakamura et al. 2003
intron 4	IVS4-2A>G	splice site	?	Boycott et al. 2001
6	c.685T>C	missense	p.Ser229Pro	Wutz et al. 2002
6	c.781G>A	missense	p.Gly261Arg	Wutz et al. 2002
7	c.832G>T	nonsense	p.Glu278Stop	Zito et al. 2003
7	c.904insG	insertion	p.Arg302fsStop	Nakamura et al. 2001
7	c.952-954delCTT	deletion	p.Phe318del	Boycott et al. 2001
8	c.1106G>A	missense	p.Gly369Asp	Strom et al. 1998, Boycott et al. 2001, Wutz et al. 2002
9	c.1218delC	deletion	p.Asp406fsStop	Bech-Hansen et al. 1998, Zito et al. 2003
10	c.1315C>T	nonsense	p.Gln439Stop	Wutz et al. 2002
13	c.1556G>A	missense	p.Arg519Gln	Strom et al. 1998
14	c.1873C>T	nonsense	p.Arg625Stop	Boycott et al. 2001, Wutz et al. 2002
15	c.2022G>A	missense	p.Gly674Asp	Nakamura et al. 2001, Boycott et al. 2001
15	c.2071C>T	nonsense	p.Arg691Stop	Zeit et al. 2005
intron 16	IVS16+2T>C	splice site	p.707-747del_ins6aa	U
17	c.2258T>G	missense	p.Phe753Cys	Wutz et al. 2002
17	c.2267T>C	missense	p.Ile756Thr	Hope et al. 2005
intron 19	IVS19-1G>C	splice site	?	Wutz et al. 2002
21	c.2579T>C	missense	p.Leu860Pro	Wutz et al. 2002
21	c.2683C>T	nonsense	p.Arg895Stop	Bech-Hansen et al. 1998, Boycott et al. 2001, Zeit et al. 2005
intron 21	IVS21+3G>A	splice site	?	Wutz et al. 2002
intron 21	IVS21-2delCA	splice site	?	Wutz et al. 2002
intron 22	IVS22+1G>C	splice site	?	Nakamura et al. 2001
23	c.2783C>A	missense	p.Ala928Asp	Boycott et al. 2001
24	c.2905C>T	nonsense	p.Arg969Stop	Strom et al. 1998, Wutz et al. 2002, Zito et al. 2003
24	c.2932C>T	nonsense	p.Arg978Stop	Nakamura et al. 2001, Nakamura et al. 2002
intron 24	IVS24+1G>A	splice site	?	Boycott et al. 2001

25	c.3006-3008delCAT	deletion	p.Ile1003del	Boycott et al. 2001
25	c.3052G>A	missense	p.Gly1018Arg	Wutz et al. 2002
27	c.3125delG	deletion	p.Gly1042del	Boycott et al. 2001
27	c.3166-3167insC	insertion	p.Leu1056fsStop	Bech-Hansen et al. 1998, Strom et al. 1998, Boycott et al. 2001, Wutz et al. 2002
27	c.3178C>T	missense	p.Arg1060Try	Strom et al. 1998, Wutz et al. 2002
27	c.3236T>C	missense	p.Leu1079Pro	Wutz et al. 2002
intron28/ exon29	IVS28-1 GCGTC>TGG	splice site	p.Cys1151Stop p.1157-1158ins5aa_Stop p.1158-1166del p.1158-1210del p.1158-1247del	II
30	c.3631-3741del111bp	deletion	p.1211-1247del	III
30	c.3673delC	deletion	p.Leu1225fsStop	Bech-Hansen et al. 1998, Boycott et al. 2001
30	c.3691-3702del12bp	deletion	p.1231-1234del	Strom et al. 1998, Wutz et al. 2002
31	c.3794G>T	missense	p.Ser1265Ile	Zeit et al. 2005
33	c.3886C>A	missense	p.Arg1296Ser	Zeit et al. 2005
33	c.3895C>T	nonsense	p.Arg1299Stop	Bech-Hansen et al. 1998, Boycott et al. 2001, Wutz et al. 2002
intron 33	IVS33+2T>C	splice site	?	Wutz et al. 2002
35	c.4075C>T	nonsense	p.Gln1359Stop	Strom et al. 1998, Wutz et al. 2002
35	c.4124T>A	missense	p.Leu1375His	Strom et al. 1998, Wutz et al. 2002
intron 35	IVS35-1G>C	splice site	?	Wutz et al. 2002
37	c.4353G>A	nonsense	p.Try1451Stop	Bech-Hansen et al. 1998, Boycott et al. 2001
38	c.4495T>C	missense	p.Cys1499Arg	Wutz et al. 2002
38	c.4499C>G	missense	p.Pro1500Arg	Wutz et al. 2002
39	c.4523T>C	missense	p.Leu1508Pro	Wutz et al. 2002
39	c.4581delC	deletion	p.Leu1527fsStop	Jacobi et al. 2003
intron 40	IVS40-2A>G	splice site	?	Boycott et al. 2001
41	c.4804A>T	nonsense	p.Lys1602Stop	Strom et al. 1998, Wutz et al. 2002
46	c.5479C>T	nonsense	p.Arg1827Stop	Wutz et al. 2002

Mutation numbering is according to NCBI nucleotide (NM_005183) and protein (NP_005174) sequences. Del, deletion; ins, insertion; fs, frameshift; aa, amino acid.

transfection control was transported properly to the plasma membrane (Figure 6A). McRory and coworkers (2004) and Peloquin and coworkers (2007) have reported that the wild type α_{1F} subunit as well as two missense mutants expressed together with the auxiliary subunits are targeted properly to the plasma membrane in HEK-293 cells. In their immunofluorescence stainings the cells, however, appear spherical, while the morphology of the HEK-293 cell growing on cover slips is known to be irregular. It may be possible that overexpression of calcium channels in the plasma membrane of HEK-293 cells leads to osmotic swelling of the cells, and therefore the morphology of the cells is altered from irregular to spherical. This hypothesis seems, however, unlikely. We also saw spherical cells in our immunofluorescence stainings (Figure 6D), but independent of the presence or absence of the auxiliary subunits, and among untransfected control cells. Also, in our experiments the spherical cells were observed only occasionally, while most of the cells expressing the α_{1F} -subunit were irregularly shaped and displayed protein aggregates. Therefore, the spherical cells most likely do

not represent cells that express channels in their plasma membrane, but may represent apoptotic, necrotic, or unattached cells.

Taken together, our results suggest that in transiently transfected cells, most of the wt $\text{Ca}_v1.4$ channel is misfolded and forms protein aggregates. Aggregate formation probably results from lack of the cell's capacity to correctly fold and translocate the α_{1F} -subunit to the plasma membrane due to its overexpression. If membrane proteins are improperly folded and translocated to the membrane, the hydrophobic side chains, which are normally embedded in the lipid bilayer, are exposed to the aqueous environment of the cytosol leading to non-native conformations that can interact to form aggregates (Johnston et al. 1998). Normally, the proteasome degradation pathway destroys the unfolded proteins, however, when the capacity of this pathway is exceeded by an increase in protein expression, protein aggregates form. In our experiments, the expression of the $\text{Ca}_v1.4$ channel protein even at lower temperatures did not yield low enough expression levels for channels to fold correctly, and proteins

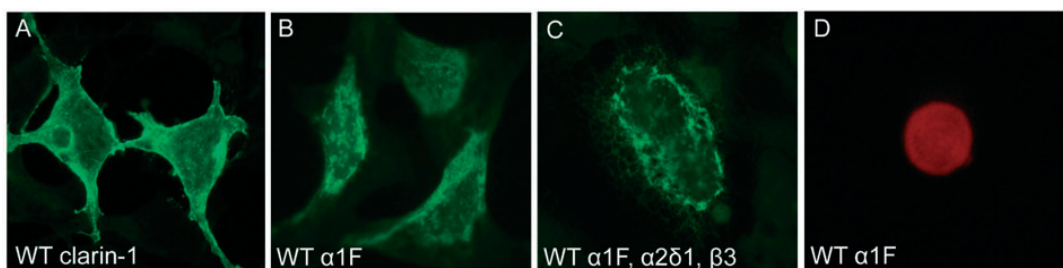


Figure 6. Subcellular localizations of wt mouse *clarin-1* and wt human $\text{Ca}_v1.4$ calcium channel in transiently transfected HEK-293 cells visualized by GFP (A) or indirect immunofluorescence with α_{1F} -antibody (B-D). Mouse wt *clarin-1* was targeted to the plasma membrane (A), whereas cells transfected with human wt calcium channel α_{1F} -subunit (B) or with human wt calcium channel α_{1F} -, $\alpha_2\delta_1$ -, and β_3 -subunits (C) formed protein aggregates in the cell. Occasionally, the morphology of the cells appeared spherical (D).

aggregated. Because the wt channel formed protein aggregates, and was not targeted properly to the plasma membrane, the effects of CORDX3 and AIED mutations on the channel targeting could not be assessed by this method. In previous studies, however, VDCCs have been overexpressed in HEK-293 cells and their function has been measured by patch clamp recordings. It is possible that while most of the overexpressed channels misfold and form aggregates, some channels may still be transported to cell membranes and the function of those channels can be studied by patch clamp recordings.

2.4.2 Comparison of CACNA1F-associated phenotypes (II, III)

All the phenotypes associated with *CACNA1F* mutations show similarities to CSNB2, the disorder in which *CACNA1F* mutations were originally identified, but some distinctive features also exist. The phenotypes of CORDX3 and CSNB2 overlap to some extent, including the range of visual acuities, myopic refraction, and the ERG abnormalities. The onset of CORDX3, however, varies from childhood to adulthood, and the disease is progressive with respect to changes in visual acuity, refraction, color vision and visual field (Mäntyjärvi et al. 2001) whereas CSNB2 is considered to be stationary and observed early in life (Allen et al. 2003; Boycott et al. 2000; Jacobi et al. 2003; Langrova et al. 2002; Nakamura et al. 2001; Pearce et al. 1990; Tremblay et al. 1995). Congenital nystagmus, hyperopic refraction, and astigmatism >1.5 D are absent from CORDX3 patients, but are relatively common in CSNB2. Also, ocular fundus changes, other than myopic changes or irregular pigmentation

in the macular area, are absent from CORDX3 patients, while CSNB2 patients may show hypopigmented fundi, and pale discs. In dark adaptation, CORDX3 patients have an elevated or missing cone threshold and a normal or only slightly elevated rod threshold, whereas in CSNB2 the cone threshold is elevated but not missing, and the rod threshold is variably elevated, from 1 to 3 log units.

CORDX3 has also many clinical features similar to other X-linked cone-rod dystrophies, CORDX1 (Brown et al. 2000; Hong et al. 1994; Jacobson et al. 1989) and CORDX2 (Bergen and Pinckers 1997; Pinckers and Timmerman 1981), including the age of onset, slightly progressive nature of the disease, the range of visual acuities, myopic refraction, ERG findings, and defects in visual fields. In CORDX1 and CORDX2, however, the fundus changes range from granular macula to bull's eye maculopathy and geographical atrophy of the RPE, whereas CORDX3 patients show only myopic changes or irregular pigmentation in the macular area. Also, patients with CORDX3 lack hyperopia and astigmatism >1.5 D, which are present in some patients with CORDX1 and CORDX2.

The phenotype of AIED patients and AIED-like patients is very similar to CSNB2. Re-evaluation of the clinical features of the patients with AIED-like disease suggests that AIED-like disease and CSNB2 are identical disorders (Wutz et al. 2002). The presence of the *CACNA1F* mutation in our AIED patients (III) indicated that also AIED and CSNB2 are allelic diseases. Furthermore, the clinical features of these diseases seem to be nearly identical. There are only a few differences between the symptoms of the patients of the original AIED

family and patients with CSNB2. AIED has progressive myopic refraction, foveal dysplasia with no foveal reflex, and a protan defect in color vision (Forsius and Eriksson 1964), whereas CSNB2 is apparently stationary with a normal fovea and mostly normal color vision, with tritan or mixed defects in some cases (Allen et al. 2003; Jacobi et al. 2003; Pearce et al. 1990). These differences are not, however, very notable, considering the high clinical variation both between and within CSNB2 families. In most CSNB2 patients, even those with same mutation, one or more of the main clinical features (night blindness, myopia, and nystagmus) is absent (Boycott et al. 2000). The most prominent feature, which is present in all patients, is impaired visual acuity.

The unique CSNB2-like disease described in a Maori family with a novel *CACNA1F* mutation has similarities to CSNB2, but is considered a distinct clinical entity (Hope et al. 2005). The patients have a more severe phenotype with color vision abnormalities. Also, intellectual disability, autism, and epilepsy are present in several male patients. Moreover, carrier females in this family have ERG abnormalities, congenital nystagmus, glare, mild nyctalopia, and decreased visual acuity often associated with high myopia. Female carrier symptoms in CSNB2 are rare: only homozygous females show clinical manifestations similar to hemizygous males (Boycott et al. 2000). The unique phenotype in the Maori family is suggested to result from gain-of-function due to an I756T mutation in the *CACNA1F* gene (Hemara-Wahanui et al. 2005). Some gain-of-function mutations, however, can lead to typical CSNB2 phenotypes (Hoda et al. 2005; Peloquin et al. 2007). Several male patients

of the Maori family also show abnormal intelligence, autism, and epilepsy (Hope et al. 2005). *CACNA1F* expression is not found in brain, but expression in pineal gland (Hemara-Wahanui et al. 2005) suggests that possible defects in melatonin production may contribute to the phenotype seen in these patients (Musshoff and Speckmann 2003; Tordjman et al. 2005). Other patients with *CACNA1F* mutations do not, however, show features of neurological defects.

Patients with a *CACNA1F* mutation which leads to retinal and optic disk atrophy (Nakamura et al. 2003) share several features with CSNB2 patients. The disease is, however, more severe with distinctive features of retinal atrophy with attenuated vessels, marked optic disc atrophy, and progressive decline of visual function resulting in poor visual acuity, while CSNB2 is stationary and patients have essentially normal fundi with only myopic changes and pale discs. Atypical retinal atrophy is also present in another CSNB2 patient with a distinct mutation in *CACNA1F* (Nakamura et al. 2002). Interestingly, the same *CACNA1F* gene mutations that are responsible for retinal and optic disc atrophy, and CSNB2 with atypical retinal atrophy, are both present also in patients with the typical CSNB2 phenotype (Boycott et al. 2001; Nakamura et al. 2001).

The variability in retinal diseases associated with *CACNA1F* mutations is evident, although all these phenotypes show some similarities to each other. Clear-cut phenotype-genotype correlation is, however, absent, which is also supported by the functional expression studies of the *CACNA1F* mutations. The reason for allelic heterogeneity is unclear.

The differences in phenotypes may, however, be caused by differences in the genetic backgrounds of patients, including the effect of possible modifier genes. This idea is supported by studies of two *Cacna1f* mutant mouse strains, which show some differences in their phenotypes even though both carry a loss-of-function mutation in the *Cacna1f* gene (Chang et al. 2006; Mansergh et al. 2005). Both of these mouse strains show absence of $\text{Ca}_v1.4$ expression, and disorganized OPL with abnormal dendritic sprouting of second-order neurons, indicating that the $\text{Ca}_v1.4$ channel is vital for normal synapse formation and/or maintenance. Other retinal layers appear normal although $\text{Ca}_v1.4$ in wt mice is expressed also in IPL. The function of $\text{Ca}_v1.4$ in IPL is unclear and may be compensated for with $\text{Ca}_v1.3$, another calcium channel expressed in retina (Xiao et al. 2007). The functional characterization of the retina indicates differences between the two *Cacna1f* mutant mouse strains. In naturally occurring *Cacna1f* null-mutant mice, nob2 (no b-wave 2), scotopic ERG shows diminished b-waves and the presence of oscillatory potentials (Chang et al. 2006), while in *Cacna1f*-knockout mice both the b-wave and oscillatory potentials are absent (Mansergh et al. 2005). In photopic conditions, the nob2 mice show reduced amplitudes (Chang et al. 2006), whereas in *Cacna1f* knockout mice the cone ERG is undetectable (Mansergh et al. 2005). Based on the ERG findings, the phenotype of nob2 mice resembles the phenotype seen in CSNB2 patients, whereas the phenotype of *Cacna1f* knockout mice is more severe with possible cone photoreceptor dysfunction, and thus more resembles the progressive retinal disorders due to *CACNA1F* mutations in humans. These phenotypic differences

observed in the *Cacna1f* mutant mice, and the fact that in humans the clinical variability of patients does not correlate with the *CACNA1F* genotype or with the functional consequences of mutant channels, indicate the involvement of other genetic and possibly also environmental and stochastic factors in the phenotypic expression of the *CACNA1F* mutations.

CONCLUDING REMARKS

Inherited retinal diseases include various different disease types that lead to partial vision loss or even complete blindness. These diseases affect millions of people worldwide, and are the leading cause of blindness among the working population in developed countries. The genetic background of a growing number of inherited retinal diseases has been identified with the development of molecular genetic techniques. As the number of known genes underlying inherited retinal diseases has grown, the presence of a vast degree of genetic and allelic heterogeneity has become more apparent. Genetic and allelic heterogeneity was observed also in this thesis, as we studied the genetic background of X-linked progressive cone-rod dystrophy (CORDX), and Åland Island eye disease (AIED). With linkage analysis in a large Finnish family, we could identify a third CORDX locus, *CORDX3*. Mutation analyses indicated that both *CORDX3* and AIED result from mutations in the L-type calcium channel gene, *CACNA1F*. In most cases mutations of *CACNA1F* lead to another retinal disease, CSNB2, but mutations may also cause other, more severe retinal phenotypes with similarities to CSNB2.

Genetic and allelic heterogeneity together with exceptional clinical heterogeneity make the diagnosis of inherited retinal diseases difficult. For example, cone-rod dystrophy may result from mutations in several distinct genes, including *CACNA1F*. Different mutations of *CACNA1F*, on the other hand, can lead to several phenotypes having few or more symptoms in common. Furthermore, the phenotypic variability can be wide even among

family members with the same *CACNA1F* mutation. Thus, a thorough clinical examination may be insufficient, and genetic tests may help to reach a correct diagnosis. In many cases, clinical examinations and family history are sufficient for successful genetic counseling of the families, including assessing the possible progression of the disease or the risk of transmitting the disease to children. Genetic studies, however, are important in carrier diagnosis and in confirming the diagnosis in patients with mild or otherwise unclear phenotypes. Genetic tests are particularly important in sporadic cases, in which the mode of inheritance can not be deduced without knowledge of the genetic defect underlying the disease. In the future, the knowledge of the genetic defect may be used in the identification of proper therapies for the patients. In the case of the *CACNA1F* gene routine genetic testing is challenging, because of the large size of the gene and lack of founder mutations or mutation hot spots. A commercial chip-based genetic test (Asper Ophthalmics) recently became available for the known *CACNA1F* gene mutations. In the case of novel mutations, however, genetic testing of patients requires sequencing of the whole gene, which is not commercially available at the moment.

No effective therapy for inherited retinal diseases exists. The molecular genetic studies, functional protein experiments, and animal models, however, advance the understanding of cellular mechanisms in health and disease, and allow insights that may eventually lead to treatment or cure.

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